VYSOKÉ UČENÍ TECHNICKÉ V BRNĚ

BRNO UNIVERSITY OF TECHNOLOGY

FAKULTA CHEMICKÁ
ÚSTAV CHEMIE MATERIÁLŮ

FACULTY OF CHEMISTRY
INSTITUTE OF MATERIALS SCIENCE

CROSSLINKING OF POLYSACCHARIDE MICROFIBERS

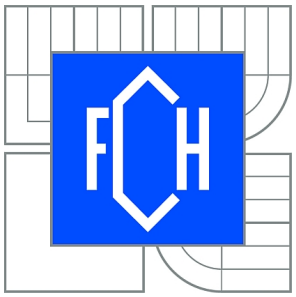
DIPLOMOVÁ PRÁCE
MASTER'S THESIS

AUTOR PRÁCE — Bc. BARBORA SVIDROŇOVÁ
AUTHOR

BRNO 2014

VYSOKÉ UČENÍ TECHNICKÉ V BRNĚ

BRNO UNIVERSITY OF TECHNOLOGY



FAKULTA CHEMICKÁ
ÚSTAV CHEMIE MATERIÁLŮ

FACULTY OF CHEMISTRY
INSTITUTE OF MATERIALS SCIENCE

CROSSLINKING OF POLYSACCHARIDE MICROFIBERS

SÍŤOVÁNÍ POLYSACHARIDOVÝCH MIKROVLÁKEN

DIPLOMOVÁ PRÁCE
MASTER'S THESIS

AUTOR PRÁCE — Bc. BARBORA SVIDROŇOVÁ
AUTHOR

VEDOUCÍ PRÁCE — Ing. LUCY VOJTOVÁ, Ph.D.
SUPERVISOR

BRNO 2014

Vysoké učení technické v Brně
Fakulta chemická
Purkyňova 464/118, 61200 Brno 12

Zadání diplomové práce

Číslo diplomové práce:	**FCH-DIP0779/2013**	Akademický rok: **2013/2014**
Ústav:	Ústav chemie materiálů	
Student(ka):	**Bc. Barbora Svidroňová**	
Studijní program:	Chemie, technologie a vlastnosti materiálů (N2820)	
Studijní obor:	Chemie, technologie a vlastnosti materiálů (2808T016)	
Vedoucí práce	**Ing. Lucy Vojtová, Ph.D.**	
Konzultanti:		

Název diplomové práce:

Síťování polysacharidových mikrovláken

Zadání diplomové práce:

1. Literární rešerše na téma hydrofilní přírodní polysaccharidy a metody jejich síťování.
2. Experimentální práce založena na přípravě chemicky síťovaných mikrovláken z polysacharidu.
3. Optimalizace podmínek za účelem získání stabilních mikrovláken ve vodném prostředí.
4. Charakterizace a porovnání mikrovláken před a po síťování.
5. Závěr

Termín odevzdání diplomové práce: 9.5.2014

Diplomová práce se odevzdává v děkanem stanoveném počtu exemplářů na sekretariát ústavu a v elektronické formě vedoucímu diplomové práce. Toto zadání je přílohou diplomové práce.

Bc. Barbora Svidroňová Student(ka)	Ing. Lucy Vojtová, Ph.D. Vedoucí práce	prof. RNDr. Josef Jančář, CSc. Ředitel ústavu

V Brně, dne 31.1.2014

prof. Ing. Jaromír Havlica, DrSc.
Děkan fakulty

Abstract

The general goal of the proposed diploma work was preparation and characterization of crosslinked hyaluronan microfibres, which would be stable in aqueous environment with proper mechanical properties.

The fibres prepared by the wet spinning technique, were crosslinked with solution containing crosslinking reagent 1-Ethyl-3-(3-dimethylaminopropyl) carbodiimide or two crosslinking reagents, 1-Ethyl-3-(3-dimethylaminopropyl) carbodiimide and *N*-hydroxysuccinimide, leading to amidation and formation of ester bonds.

Characterization of prepared samples is the main goal of experimental part of the thesis. The swelling characteristics were carried out to determine the stability of fibres in three different solutions (with pH 7.4, 3 and 11). For determination of thermal stability was used thermogravimetrical analysis, and for determination of esterification and amidation was used infrared spectroscopy with Fourier transformation. Mechanical properties of fibres were studied by stress-strain tester. Additionally, the rheological properties were investigated, as well as the microstructure and surface of fibres by scanning electron microscopy.

The fibres before chemical crosslinking had lower stability in all three types of solutions; the thermal stability was also lower, than the stability of crosslinked fibres. For not chemically modified fibres with crosslinking reagent, only one type of peak for esterification occurred. The infrared spectra of chemically crosslinked fibres showed the presence of two esterification peaks, which was the result of the effectiveness of the crosslinkig reagent. Amidation was also stronger for crosslinked fibres, especially for fibres crosslinked long time and in the solution with higher concentration of crosslinking reagent. Due to the heterogeneity of fibres, mechanical properties did not show evidence of any dependence on the crosslinking. The rheological study showed that the viscosity of fibre dissolved in water is less dependent on the shear rate than the powder of sodium hyaluronan dissolved in water.

Despite more different methods of characterization of fibres which were used in this work, there are still many options for better characterization and closer understanding of this biopolymeric material.

Keywords

biopolymers, hyaluronic acid, microfibers, crosslinking

Abstrakt

Všeobecne, cieľom tejto diplomovej práce bola príprava a charakterizácia sieťovaných hyaluronových mikrovláken, ktoré by boli stabilné vo vodnom prostredí s vhodnými mechanickými vlastnosťami.

Vlákna pripravené technikou zvlákňovania z roztoku boli sieťované pomocou roztoku so sieťovacím činidlom 1-Ethyl-3-(3-dimethylaminopropyl) carbodiimidom alebo roztoku s dvoma sieťovacími činidlami 1-Ethyl-3-(3-dimethylaminopropyl) carbodiimidom a *N*-hydroxysuccinimidom, vedúcim k amidácii a tvorbe esterových väzieb.

Charakterizácia pripravených vzoriek je hlavným cieľom experimentálnej časti práce. Charakteristiky botnání boli vykonané na určenie stability vláken v troch rôznych roztokoch (s pH 7.4, 3 a 11). Na určenie termickej stability bola využitá termogravimetrická analýza a na stanovenie esterifikácie a amidácie bola použitá infračervená spektroskopia s Fourierovou transformáciou. Mechanické vlastnosti vláken boli študované pomocou testovania závislosti stress-strain. Ďalej boli testované reologické vlastnosti ako aj mikroštruktúra a povrch vláken pomocou skenovacieho elektrónového mikroskopu.

Vlákna pred chemickým sieťovaním vykazovali nižšiu stabilitu vo všetkých troch roztokoch, termálna stabilita bola taktiež nižšia ako stabilita zosieťovaných vláken.

Pre vlákna chemicky nemodifikované so sieťujúcim činidlom, sa objavil iba jeden typ píku pre esterifikáciu. Infračervené spektrum chemicky zosieťovaných vláken ukázalo prítomnosť dvoch píkov pre esterifikáciu, čo je prejavom efektivity sieťovacieho činidla. Amidácia bola tiež výraznejšia pri zosieťovaných vláknach, špeciálne pre vlákna sieťované dlhú dobu a v roztoku s vyššou koncentráciou sieťujúceho činidla. Kvôli nerovnomerným vláknam, mechanické vlastnosti nevykazovali žiadnu závislosť na sieťovaní. Štúdium reológie ukázalo, že viskozita vlákna rozpusteného vo vode je menej závislá na šmykovej rýchlosti ako prášok hyaluronanu sodného rozpusteného vo vode.

Napriek mnohým rôznym metódam charakterizácie vláken, ktoré boli použité v tejto práci, je stále veľa možností pre lepšiu charakterizáciu a bližšie pochopenie tohto biopolymérneho materiálu.

Klíčová slova

biopolyméry, hyaluronová kyselina, mikrovlákna, sieťovanie

SVIDROŇOVÁ, B. *Síťování polysacharidových mikrovláken.* Brno: Brno University of Technology, Faculty of Chemistry, 2014. 92 p. Supervisor Ing. Lucy Vojtová, Ph.D.

Declaration

I declare that the diploma thesis has been worked out by me and that all the quotations from the used literary sources are accurate and complete. The content of the diploma thesis is the property of the Faculty of Chemistry of Brno University of Technology and all commercial uses are allowed only if approved by both the supervisor and the dean of the Faculty of Chemistry, VUT.

……
Student's signature

Acknowledgement

I would like to thank my supervisor Ing. Lucy Vojtová, Ph.D. for giving me the opportunity to work on this thesis and for her advice. I also thank to M.Sc. Abdel-Mohsen Abdel-Lattif, Ph.D. for his help and professional advice. I would also like to thank all people who helped me with dealing with the work and problems all the way along.

Content

1 INTRODUCTION

Inspired in the nature, in the formation of fibres in plants, man-made fibres are important form of materials. [1] In history, artificial fibrous structures in the form of textiles were used as clothing and decorations. In the past century, fibre-based techniques have been widely used in numerous engineering applications such as filtration, composite fabrication, energy systems, and micro-fluidics. Recently, some of the fibre-based techniques have been applied for tissue engineering for preparation of scaffolds. [2] Fibrous scaffolds normally have large surface area and volume, homogeneous fibre size and a wide range of pore distribution. [3]

In tissue engineering and medicine, biopolymers are widely used due to their similarities with the extracellular matrix, high chemical versatility, typically good biological performance and inherent cellular interaction, and the cell or enzyme-controlled degradability. [4] These materials allow supplement, or replace the functions of living tissues of the human body. [5]. One of these naturally occurring biopolymers with important biological functions is hyaluronic acid. This high molecular weight polysaccharide is widely used in many medical fields, from different types of surgeries to wound healing. In the tissue engineering, mentioned ability of wound healing is the desirable property of hyaluronic acid, where the degradation products of hyaluronic acid are able to modulate wound healing. [6][7]

Combining the advantages of fibres with the properties of hyaluronic acid, wound dressings can be a proper result. Wound dressings are used to encourage wound healing and create better healing conditions. Hyaluronic acid can also be used as a carrier for other wound healing agents. [8]

The main aim of the work is to crosslink hyaluronan fibers by using 1-Ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC) with/without *N*-hydroxysuccinimide (NHS) for the first time. The second aim of study is characterization of prepared fibres and their comparison.

2 THEORETICAL PART

2.1 Fibres

Fibres can be referred to as oriented polymers and in general, fibres are at least 100 times longer than they are wide. [9][10] They are an important form of polymer materials; they are of great practical importance in creating domestic objects (clothing, carpets, etc.) or technical agents (medical articles, insulating materials, etc.), but can also be found in animal and plant world (skeletal structures, connective tissues, skin and protective membranes, etc.). [1]

The properties of fibres are function of many factors, primarily the chemical composition of the polymer and the physical state of the polymer in the fibre, i.e., its structure. [12] A number of properties of man-made polymer fibres are associated with natural fibre formation. These shared properties of natural and man-made polymer fibres include mechanical (strength and deformational) structural (relationship between crystalline, interphase, and amorphous state) characteristics, the nonequilibrium physical state and relaxation phenomena, and the effect of the chemical structure of the polymer material on the stability of the fibres in time. [1]

Despite the difference in the properties of different polymer fibres, all the fibres have common structural properties that determine their mechanical, thermal, and other properties. [9] Two basic types of structures occur in fibres:

Macrostructure - structural regions are comparable to the transverse dimensions of a fibre. It includes segments with different density, porosity, orientation of the polymer chains, crystallinity, etc. The size of the segments with a different structure along a fibre can vary from several micrometers to 10 m and more – these are layers, pores, thickened and thinned parts, etc.

Microstructure – means the supramolecular formations of the polymer, crystallites with different longitudinal and transverse dimensions, and the reciprocal position and state of the intercrystallite (amorphous) phase of the polymer. The size of the formations ranges from several tens of angstroms to up to 100 Å.

The structure of the polymer in the fibre is established as it is fabricated in all stages of the process chain, from synthesis of the polymer to textile operations. [12]

Articles made of fibre materials, such as drains, wound coverings, retaining bandages, etc., are widely used in medical practice. [13]

The process of preparing fibres is called spinning. [10] The following chapters provide a description of some basic types of spinning.

2.1.1 Wet Spinning

The process of wet spinning involves extrusion of a polymeric liquid through fine holes known as spinnerets. The polymer solution is spun into a coagulating solution to precipitate the polymer. This solution draws out the solvent and leaves behind only the polymer. The resulting fibres are oriented and the degree of crystallinity is increased. This has the effect of increasing the modulus and tensile strength of the fibres. This technique is required for polymers that require dissolving in a solvent to be spun. [14], [15] By wet spinning are prepared fibres for general household applications and also a different kind of industrial fibres (e.g. thermostable, heat-resistant). [12] This method is also suitable for natural polymers, for example, collagen, silk fibroin or chitosan. [16], [17], [18]

2.1.2 Electrospinning

Electro-spinning is a technology that enables the production of continuous fibres with dimensions in the range of nanometres to a few microns. These fibres possess high surface area to volume ratio, high porosity similar to the natural extracellular matrix (ECM), and other outstanding properties. Electro-spun fibres can be oriented or arranged randomly. [19], [20], [21]

In process of electro-spinning, an electrical potential is applied between a droplets of a polymer solution and held at the end of the nozzle of the spinneret and a grounded collector plate. When the applied electric field overcomes the surface tension of the droplet, a charged jet of polymer solution, which is controlled by the electric field, is ejected. The ejected fibre jet is known as the Taylor cone. The ejected jet has bending instabilities caused by repulsive forces between the charges carried with the jet. The jet grows longer and thinner until it is collected on the collector plate as fibres. [21], [22]

A variety of material can be used for electro-spinning including: biodegradable, non-degradable, natural and synthetic materials. For example, polymers used for electro-spinning, are alginate, cellulose, chitin, hyaluronic acid. [19], [21] The uses of electro-spinning can be found in various applications such as wound healing, scaffolds in tissue engineering, drug delivery, enzyme immobilization, biosensors, filtration, cosmetics, etc. [22]

2.1.3 Dry spinning

It is a process, in which a mixture of solvents and polymer is extruded through a die into a warm gas. As the material moves through the gas, it is heated and the solvents evaporate. The concentration of polymer increases and the jet solidifies into a fibre that is taken up by a roller at a speed higher than the extrusion speed. [23]

Dry spinning is used to produce man-made fibres from polymers such as cellulose acetate, cellulose triacetate, polymers and copolymers of vinyl chloride, and acrylonitrile. [24]

2.1.4 Melt spinning

Polymers that melt on heating without undergoing any decomposition are spun by a melt spinning system. [25] The molten polymer is extruded through spinnerets in a vertical chamber where the molten filaments are cooled with circulating air. Following the spinning stage, the extruded tow of filaments is subjected to a high degree of stretching to align the constituent polymers. This way the material achieves the required fibre fineness and usable tensile properties. [26] Typical polymers spun this way are polyamide 6, polyamide 6.6, and polyester fibres (poly(ethylene terephthalate)). [27] Fibres of biopolymer poly(3-hydroxybutyrate) were also prepared by this technique. [28]

2.1.5 Biopolymers for spinning techniques

Just like the nature has used the biological polymers as the material of choice, biopolymers can be the choice for fabrication new materials for man, also in the form of fibres, thanks to their properties and great possibility in application in medical field. [2], [29] Combination of fibres technology and medical sciences has resulted into a field called medical textiles. [30] Textile materials are suitable for many medical and surgical applications where a combination of strength and flexibility are required. [31]

2.2 Biopolymers

Biopolymers are polymers formed in nature during the growth cycles of all organisms; hence, they are also referred to as natural polymers. [32] These polymers offer the advantage of being similar to biological macromolecules, therefore the biological environment is prepared to recognize them and deal with these polymers metabolically. One of the many important properties of biopolymers is the similarity with the extracellular matrix (ECM). [33] They are generally non-toxic, even at high concentrations; biocompatible and biodegradable. On the other hand, many biopolymers have poor mechanical properties and are limited in supply, and can therefore be costly. [34], [35]

Natural polymers can be obtained by the fermentation of micro-organisms, produced in vitro by enzymatic processes, the largest amount is extracted from plant. Living organisms are able to synthesize a variety of polymers. These polymers are divided into major classes according to their chemical structure: polysaccharides, proteins and polyesters. [33]

Polysaccharides are polymeric carbohydrate structures, formed of repeating units (either mono- or di-saccharides) joined together by glycosidic bond. The structures are often linear, but may contain various degrees of branching. [35] The example of polysaccharide can be cellulose, starch, xanthan or hyaluronic acid.

Proteins are specific copolymers with regular arrangements of different types of α-amino acids. Proteins are e.g. collagen, wool or silk. [32]

Polyesters are found in nature in a wide range of bacteria, higher plants and are also produced naturally in some animals. Example of polymer produced within the bacteria is poly(3-hydroxybutyrate) and shellac is polymer produced by animal. [37]

Biopolymers can be used in traditional commodity, in agriculture, filtration, hygiene and protective clothing. However, the main application is in the medical field. The medical application include drug delivery systems, wound closure and healing products, surgical implant devices, and scaffolds for tissue engineering. [29]

2.2.1 Biopolymer for spinning: Hyaluronic Acid

Hyaluronic acid, as a biopolymer, is thanks to its great chemical and biological properties, natural appearance in a human body, and the possibility of obtaining it from various natural sources a very attractive subject for biotechnology, and medicine in general. Hyaluronic acid is an important material in wound healing, for example Laserskin®, which is maximally-esterified hyaluronic acid with perforations or it can serve as an anti-inflammatory component in the wound dressing materials. [7], [38]

The combination of spinning techniques and hyaluronic acid can bring new possibilities in preparation of new types of wound dressing materials.

2.3 Hyaluronic acid

Hyaluronic acid (HA, hyaluronan, sodium hyaluronate) is a naturally occurring biopolymer with unique advantages, it is easy to produce and modify, and it is used across a wide variety of medical fields and in cosmetic practice.

Hyaluronan belongs to a group of substances known as glycosaminoglycans (GAGs), being structurally the most simple amongst them, and the only non-sulfated one. [7], [38], [39]

2.3.1 Properties and structure of hyaluronic acid

Hyaluronic acid (HA) is a linear and anionic polymer which consists of two modified sugars, D-glucuronic acid and *N*-acetyl-D-glucosamine (Figure 1). They are linked together through alternating beta-1,4 and beta-1,3 glycosidic bonds. [38] Both sugars are spatially related to the glucose which in the beta configuration allows all of its bulky groups: the hydroxyls, the carboxylate moiety and the anomeric carbon on the adjacent sugar, to be in sterically favourable equatorial positions. At the same time, all of the small hydrogen atoms occupy the less sterically favourable axial positions. For this reason, the structure of the disaccharide is energetically very stable. [40] The functional groups (carboxylic acids and alcohols) along the backbone can be used to introduce the functional domains or to form a hydrogel by crosslinking. [41]

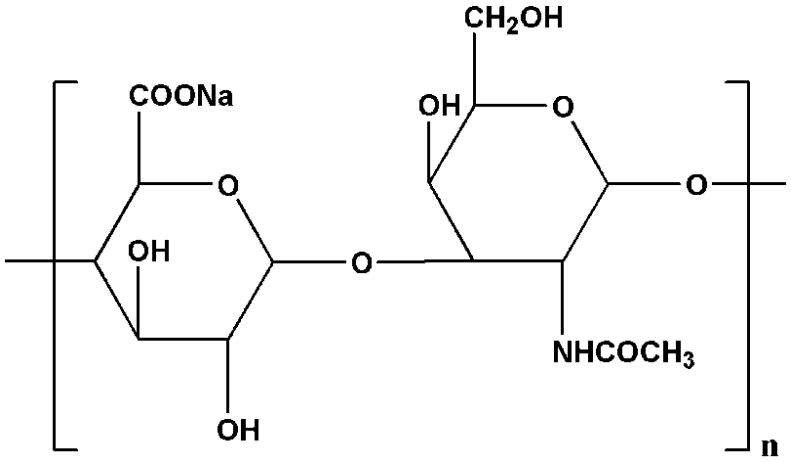


Figure 1 *Structure of the disaccharide repeating unit of hyaluronic acid. [38]*

In aqueous solution, HA forms tertiary structures β sheets based on 2-fold helixes anti-parallel hyaluronan chains. Sphere occupied by HA molecule is quite large, but not impenetrable. Therefore, HA forms specific overlapping domains creating meshwork which is stabilized by specific H-bonds (up to five H-bonds per tetrasaccharide unit of HA), water bridges and hydrophobic interactions. The actual mass of hyaluronan within this domain is very low, and small molecules such as water, electrolytes and nutrients can diffuse freely through solvent, within the domain. Large molecules such as proteins will be partially excluded from the domain because of their hydrodynamic sizes in solution. [42] Hyaluronan's polarity and the formation of such a meshwork is a potential reason for the higher osmotic pressure in solution which is the cause of hyaluronan's high water retention capacity. [43], [44]

HA in aqueous solution undergoes a transition from Newtonian to non-Newtonian characteristics with increasing molecular weight, concentration or shear rate. [45]

HA is in the form of sodium salt in physiological conditions, because at pH 7, the carboxyl groups are predominantly ionized. The hyaluronan molecule is a polyanion that has associated exchangeable cation counterions to maintain charge neutrality. [42] In this case is HA referred to as sodium hyaluronate. [46] The backbone of a hyaluronan molecule in a physiological solution is stiffened by a combination of the chemical structure of the disaccharide, internal hydrogen bonds, and interactions with solvents. The preferred shape in water features hydrophobic patches on alternating sides of the flat, tape-like secondary structure. [40], [42]

HA has a range of molecular sizes from 1000 to 10,000,000 Da with the average length of a disaccharide approximately 1 nm. However, the molecular weight depends on the source of HA. [47], [40], [45]

Hyaluronic acid is enzymatically degraded by enzyme hyaluronidase and is completely resorbable through multiple metabolic pathways. [39] Enzymatic degradation cleaves the HA macromolecule into HA fragments of precisely defined size for the desired biological function. HA can also be naturally degraded in the organism by reactive oxygen species. [46] As a very large molecule, hyaluronan is prone to

mechanical degradation either by ultrasonic treatment or by thermal degradation at elevated temperatures. [42]

When HA is not bound to other molecules; it binds to water and forms highly viscous solutions. [40], [48] Hydration and/or water holding capacity is one of the most important aspects of the HA function. [49] HA can immobilize water in tissue and thereby change dermal volume and compressibility. [38]

It has several important biological functions in mammal bodies. In the body, it plays a critical role as a signalling molecule in cell motility, cell differentiation, wound healing. Native HA degrades rapidly and resides within the tissue only for 1-2 days. [40], [43], [50], [51] HA shows some pH sensitivity in aqueous environment, especially when it is cross-linked to produce drug delivery systems like hydrogels or micro (or nano) particles. [16]

Large matrix polymers of HA are space-filling, anti-angiogenic, and immunosuppressive. The intermediate-sized polymers, which consist of 25-50 disaccharides, are inflammatory, immunostimulatory, and highly angiogenic. Smaller oligosaccharides are antiapoptotic and induce heat shock proteins. [38]

Hyaluronic acid however possesses poor mechanical properties. [51]

2.3.2 Synthesis, production

Commercially produced HA is isolated either from animal sources, within the synovial fluid, umbilical cord, skin, and rooster comb, or from bacteria through a process of fermentation or direct isolation. [45]

2.3.3 Occurrence of HA

Hyaluronic acid is almost omnipresent in the human body and in other vertebrates; it is also present in the capsules of some bacteria. HA is absent in fungi, plants and insects. It primarily occurs in the ECM and pericellular matrix, it is also a major intracellular component of connective tissues such as skin, the synovial fluid of joints, vitreous fluid of the eye, the scaffolding within cartilage and the umbilical cord.

Significant amounts of HA are also found in lung, kidney, brain, and muscle tissues. [40] The highest content of HA in the human body is in the synovial fluid, umbilical cord, and vitreous fluid of the eye.

By far, the largest content of HA is found in rooster combs. [38], [52] The concentration of HA in rooster comb is 7.5 $mg \cdot mL^{-1}$, in the synovial fluid 3-4 $mg \cdot mL^{-1}$, in umbilical cord 3 $mg \cdot mL^{-1}$, in the vitreous humor of the eye 0.2 $mg \cdot mL^{-1}$, and in skin 0.5 $mg \cdot mL^{-1}$. The largest deposit of hyaluronan resides in the skin; in an adult human this totals ~ 8 g. [42]

2.3.4 Medical Application

Preparations of hyaluronan or higher crosslinked products are used for many medical applications. [42]

2.3.4.1 Wound healing and scarring

As the HA is naturally present in the skin and soft connective tissues, it is an appropriate choice for a matrix to support dermal regeneration and augmentation. [40] Hyaluronic acid is also involved in the process of wound repair. During wound healing, HA promotes epithelial migration and differentiation, improves angiogenesis, and enhances collagen production. A 100 % benzyl-esterified derivative of hyaluronic acid processed into fibres and prepared as a flat, non-woven pad dressing, which can be seeded with fibroblasts before application was manufactured. [53] According to Prestwich and co-workers, crosslinked HA gel films accelerate the healing of full-thickness wounds, presumably by providing a highly hydrated and nonimmunogenic environment. HA scaffolds have been also used in burn care or for difficult-to-heal wounds. [40]

2.3.4.2 Adhesion prevention

Most surgical procedures are accompanied by undesired tissue damage, which can lead to abdominal tissue adhesion. It is a complex inflammatory condition that can cause pain and impact organ function. For example, a new, internally-esterified material, the HA autocrosslinked polymer hydrogel, is effective in reducing postsurgical adhesions in laparoscopic surgery. [42], [47], [54]

2.3.4.3 Drug delivery

HA is usually conjugated or coated with the chemical drug or polymer to enhance therapeutic efficiency as well as to provide sustained release properties. For example, HA has been used for targeting specific intracellular delivery of genes or anticancer drugs. The HA has also found an application in ocular, nasal or parenteral delivery. It is used in ocular delivery because of its nonirritating property and high water-binding capacity. The absorption of drugs and proteins via mucosal tissues could be enhanced thanks to the mucoadhesive properties of HA in the nasal drug delivery systems. [55] Generally, HA is thought to act as either a mucoadhesive and retain the drug at its site of action/absorption or to modify in vivo release/absorption rate of the therapeutic agent. [45] HA can be combined with partner molecules (such as isopropylacrilamide, polyacrylic acid or cellulose) and different biophysical properties for various applications can be obtained. [7]

2.3.4.4 Orthopedic surgery

HA provides necessary lubrication for the joint and serves as shock absorber. It reduces the friction of the moving bones and diminishes wear of the joint. [38] Administration of purified high molecular weight HA into orthopaedic joints can restore the desirable rheological properties and alleviate some of the symptoms of osteoarthritis. [45] A HA gel has been successfully trialled as a carier mechanism for antibiotics to the eye and injection of chondroitin sulphate and HA mixture prevents endothelial damage from air bubbles created intraoperatively. [7]

2.3.4.5 Ophthalmics

HA is commonly used in ophthalmology for the dry eye treatment and as a viscoelastic device in ophthalmologic surgery owing to the ability to form highly viscous solutions even at low concentrations. [56] During the ophthalmologic surgery (*cataract surgery* or *deep lamellar keratoplasty*) intracameral injection of viscoelastic HA can maintain anterior chamber length and protect the corneal endothelium. [57]

2.3.4.6 Other medical applications

Hyaluronic acid is also used in clinical medicine as a diagnostic marker for many diseases including cancer, rheumatoid arthritis and liver pathologies. [38] In form of implants, as a filling material, it is used to treat conditions as urinary incontinence, otolaryngology or reconstructive surgery. HA based scaffolds in combination with other materials have been investigated, for example freeze dried chitosan/HA, freeze dried HA/collagen scaffolds, or freeze dried glutaraldehyde crosslinked HA/polyglycolic acid scaffolds. These scaffolds were prepared for bone and cartrilage repair and regeneration. [41]

2.4 Crosslinking

Crosslinking is process of intermolecular binding, in which previously separated molecules are bonded with permanent chemical bonds. The crosslinking firstly results in a slight increase in molecular weight, and then forms a network. The crosslinking can modify many of the physical properties of a polymer. Especially the mechanical properties of long chain polymers depend not only on chemical structure and chain mobility, but also on the association between adjacent molecules. [58]

Polymeric materials can be divided into two main categories, dependent on their molecular weight: polymers with molecular weight higher than about 10^5 $g \cdot mol^{-1}$ and polymers which have low molecular weights, lower than 10^4 $g \cdot mol^{-1}$, often in the range $2\text{-}3 \cdot 10^3$ $g.mol^{-1}$. Polymer materials in the lower molecular-weight range often require a crosslinking step to obtain satisfactory mechanical properties.

Different types of crosslinking are possible:

Covalent crosslinking, which is regarded as the most stable;

Ionic bonds;

Physical crosslinking, via Van der Waals, hydrogen bonds, or other interactions. [59]

Physical and ionic crosslinking occurs in solutions of high molecular weight HA. These two crosslinking mechanisms are difficult to control, which limit the final network structure and properties. [60], [61]

2.4.1 Chemical crosslinking

In general, it is the process of chemically joining two or more molecules by a covalent bond. Modification involves attaching or cleaving chemical groups to alter the solubility or other properties of the original molecule. [62]

The crosslinking reactions have been accomplished under acidic, neutral, and alkaline conditions. [41]

2.4.1.1 Crosslinking reagents

Crosslinking reagents contain two or more reactive ends that are capable of attaching to specific functional groups on molecules [62] Crosslinkers can be divided in two groups: homobifunctional crosslinkers, and hetrobifunctional crosslinkers. Homobifunctional crosslinkers are molecules that have the same reactive groups on each end of the crosslinker. These crosslinkers can also create intramolecular crosslinks. Heterobifunctional crosslinkers are molecules that have different reactive groups on each end of the crosslinker. They are more selective and work in a two-step process. [63]

2.5 Crosslinking of HA

When HA is crosslinked, different HA chains are linked together by two bonds or more, as shown in Figure 2. Crosslinking procedures can be: direct crosslinking, crosslinking of HA derivates and crosslinking of different HA derivates. [46] The density of prepared HA network depends on the degree of crosslinking that is achieved. [64] Hyaluronan has four reactive groups: acetamido, carboxyl, hydroxyl and the reducing end. These groups have the ability for crosslinking to itself or other polymers. [65] The main responsibility for crosslinking of HA molecules have the hydroxyl and carboxyl functional groups. Hydroxyl groups may be crosslinked via an ether linkage and carboxyl groups via an ester linkage. The process of crosslinking is the most common modification of hyaluronan to form a hydrogel.

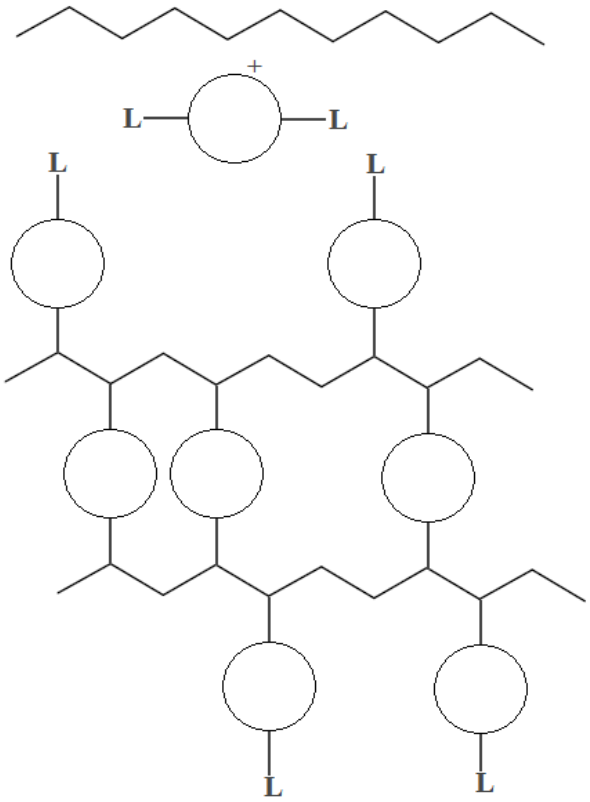


Figure 2 *Chemical crosslinking of polymer. [46]*

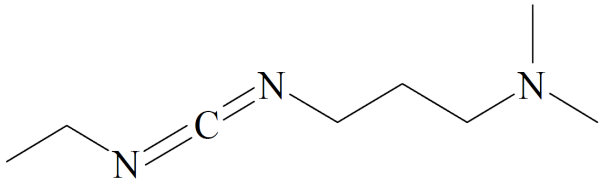
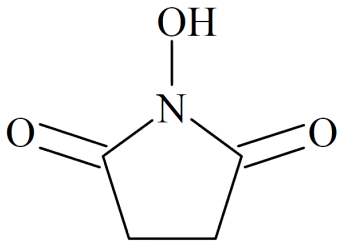
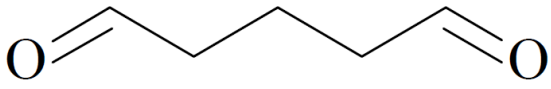
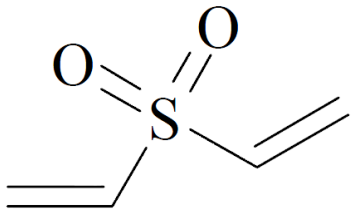
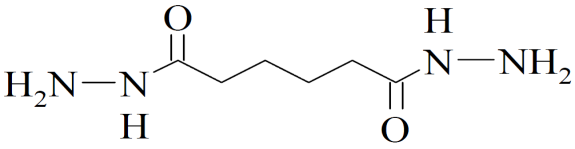
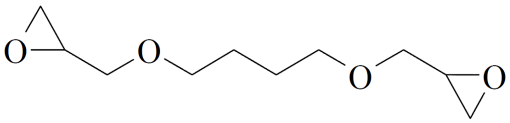
HA may be treated with acid or base and this treatment will results in at least partial deacetalization, resulting in the presence of free amino groups.

Amino groups may be crosslinked via an amide (-C(O)-NH-); imino (-N=CH-) or secondary amine (-NH-CH-) bond. An imino linkage can be converted into an amino linkage in the presence of a reducing agent. [66]

Since HA is soluble in water, crosslinking can be performed in aqueous conditions. However, some reactions are pH-dependent and need to be performed in acidic or alkaline conditions, which induce significant HA chain hydrolysis. [46]

The agents already used for crosslinking of HA are 1-Ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC), *N*-hydroxysuccinimide (NHS), glutaraldehyde (GTA), divinyl sulfone (DVS), poly(ethyleneglycol) diglycidyl ether (PEGDG), butanediol-diglycidyl ether (BDDE) and many others. [46], [67], [68], [69] Formulas of these reagents are in the Table 1. General crosslinking agents for HA chains usually include carbodiimides, hydrazides, aldehydes, sulfides, and polyfunctional epoxides. Chemical modification of HA enables the polysaccharide to be used for various biomedical applications. [64]

Table 1 *Chemical formulas of the crosslinking reagents. [70], [71],[72], [73], [74]*

EDC	NHS
	
GTA	**DVS**
	
ADH	**BDDE**
	

2.5.1 1-Ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC)

EDC is a water-soluble, highly efficient “zero-length” crosslinker, which does not bind to HA molecules; it just activates the carboxyl group of HA. [46], [75], [76] EDC reacts with carboxylic acid groups to form an active O-acylisourea intermediate, which is very short-lived and readily undergoes rearrangements. The second step is the nucleophilic attack by the amine on the activated HA. An amide bond is formed between the primary amine and the original carboxyl group. An EDC by-product is released as a soluble urea derivate. The O-acylisourea intermediate is unstable in aqueous solutions, it reacts with water and it quickly rearranges into a stable N-acyl urea byproduct. The intermolecular formation of ester bonds between the hydroxyl and carboxyl groups of HA is observed. [46], [76], [77] In general, EDC is preferable among the crosslinking reagents because it can induce crosslinking of biomaterials simply by changing them into water-soluble urea derivates that have very low cytotoxicity. [78] The crosslinking reaction is shown in Figure 3.

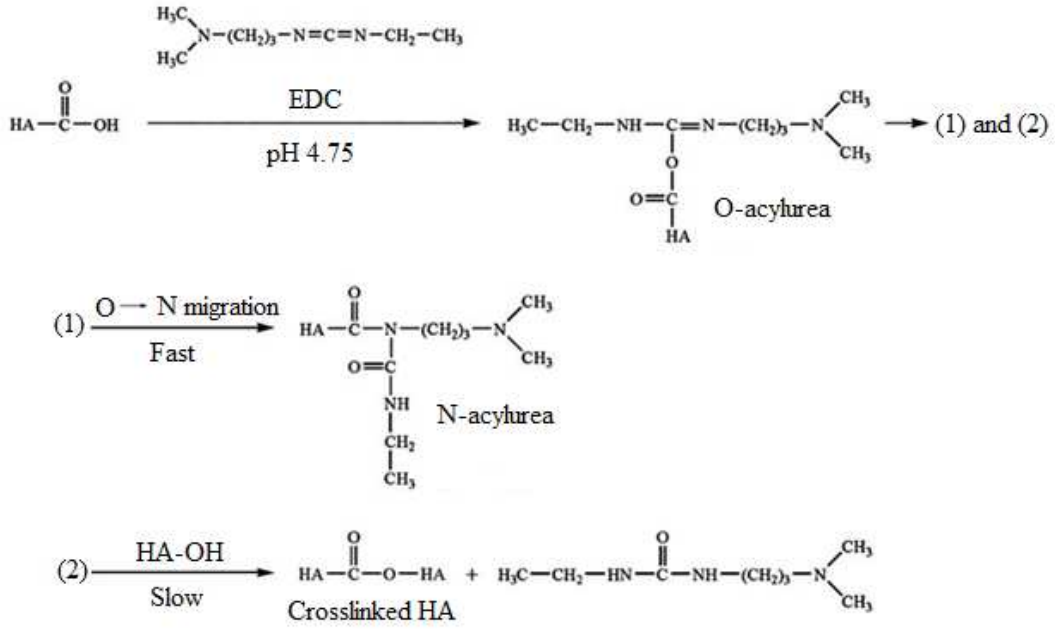


Figure 3 *Crosslinking reaction scheme of HA with EDC. [76]*

2.5.2 *N*-hydroxysuccinimide (NHS)

NHS is a homobifunctional one of the most common used crosslinker. [79] This reagent is used with EDC reagent in order to prevent the formation of the irreversible N-acylurea by-product (Figure 4). The use of EDC together with NHS causes a formation of more hydrolysis-resistant and non-rearrangeable intermediates. [46] HA-N-succinimide is an activated ester of the glucuronic acid moiety and this HA-active ester intermediate is a precursor for crosslinked hydrogels. [80]

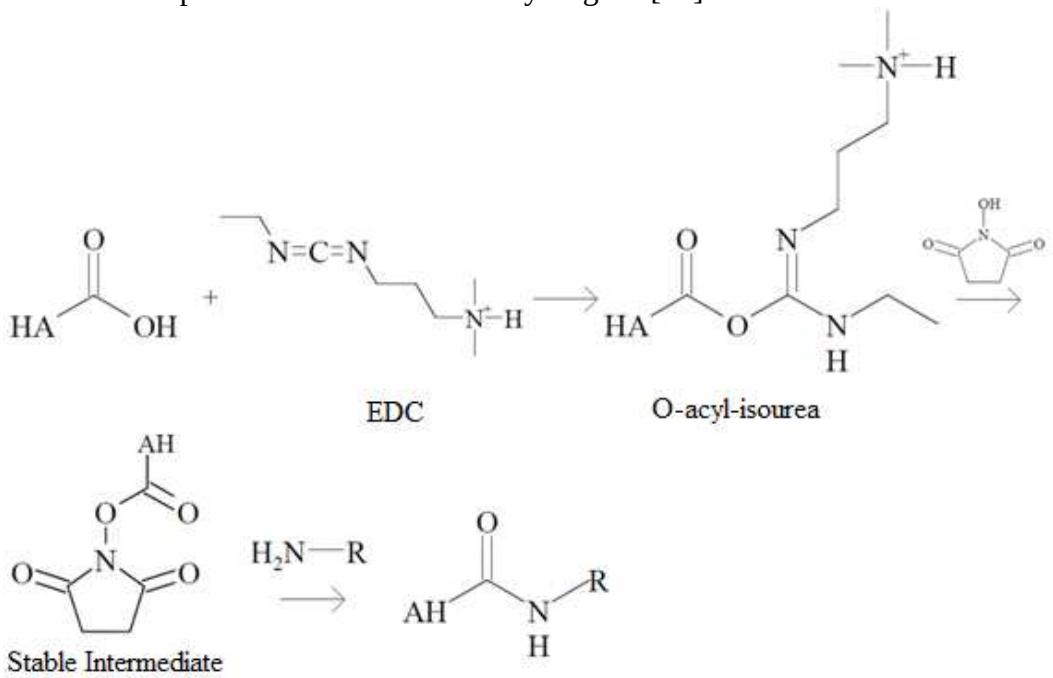


Figure 4 *Crosslinking of HA with EDC and NHS. [46]*

2.5.3 Glutaraldehyde (GTA)

Glutaraldehyde is in water soluble crosslinker ans it is believed to form either a hemiacetal or an ether link with HA under acidic conditions (Figure 5). Glutaraldehyde has been widely use for crosslinking of proteins such as collagen and gelatine, but it has been found out that HA readily undergoes crosslinking with GTA when a film of HA is subjected to the crosslinking reaction. [66], [81] However, GTA is generally considered to be toxic, thus it requires specific handling during the reaction and purification of final product. [46], [82]

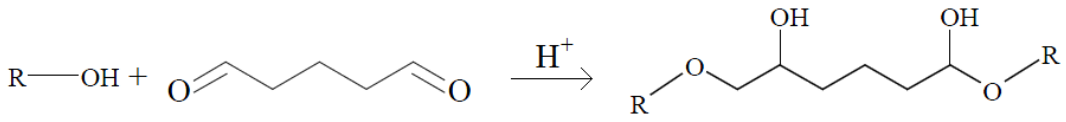


Figure 5 *The OH group on the hyaluronic acid reacts under acidic conditions with glutaraldehyde giving hemiacetal or ether ether crosslinks. [66]*

2.5.4 Divinyl sulfone (DVS)

DVS crosslinker causes the occurance of the crosslinking via the hydroxyl groups forming an ether bond (Figure 6). [66] The hydroxyl groups of the HA chains react to form an infinite network of sulfonyl bis-ethyl crosslinks. DVS reacts readily with HA in aqueous alkaline solutions at room temperature. The reaction is fast, and strong gels are obtained within minutes. DVS crosslinking does not involve the biologically reactive functional groups on the HA molecule, thus the prepared gels largely retain the biological properties of native HA. Hyaluronic acids which are chemically crosslinked with DVS are called hylans. [41], [83]

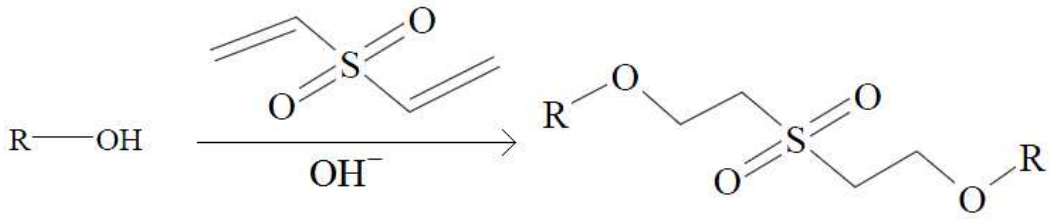


Figure 6 *The OH group on the hyaluronic acid reacts under alkaline conditions with DVS to give sulgonyl bis-ethyl crosslinks. [66]*

2.5.5 Adipic dihydrazide (ADH)

ADH is a water-soluble homobifunctional crosslinikng reagent that provides 10-atom bridge between crosslinked molecules. [84] Covalent attachment of this reagent to the carboxylic acid groups of HA under mild conditions results in the availability of pendant hydrazide amino functionalities arrayed along the hyaluronate backbone. [85] The reaction of HA with this crosslinking reagent is shown in Figure 7.

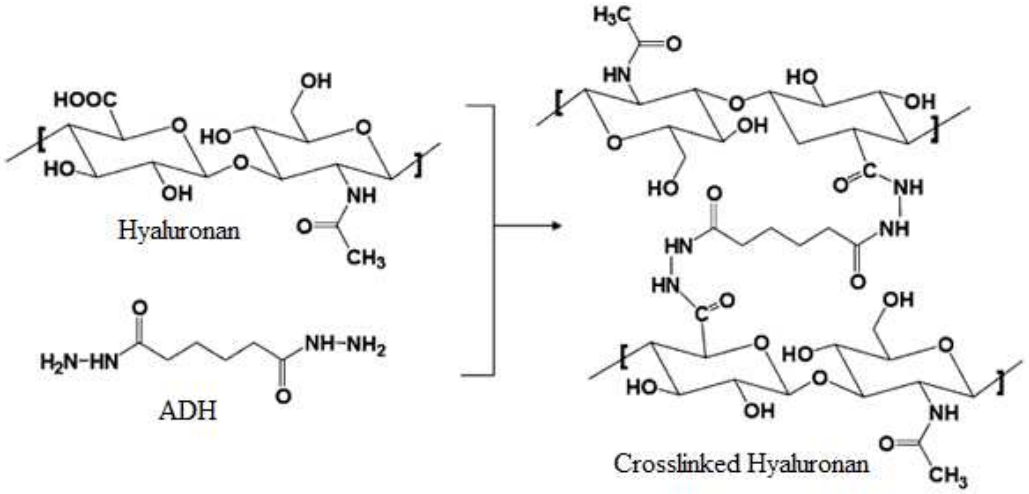


Figure 7 *Illustration of crosslinking reaction of hyaluronan with adipic dihydrazide. [86]*

2.5.6 Butanediol-diglycidyl ether (BDDE)

BDDE is a bifunctional crosslinker that reacts with HA's primary hydroxyls. The reaction consists of the epoxide ring opening to form ether bonds with the HA hydroxyl groups. Crosslinking is accomplished simply by mixing HA with BDDE in water and appropriately adjusting the temperature and pH. [46], [87] This crosslinking reagent is already used in commercialized formulations of HA as dermal filler. [88] Figure 8 shows reaction of BDDE with HA in alkaline and acidic conditions.

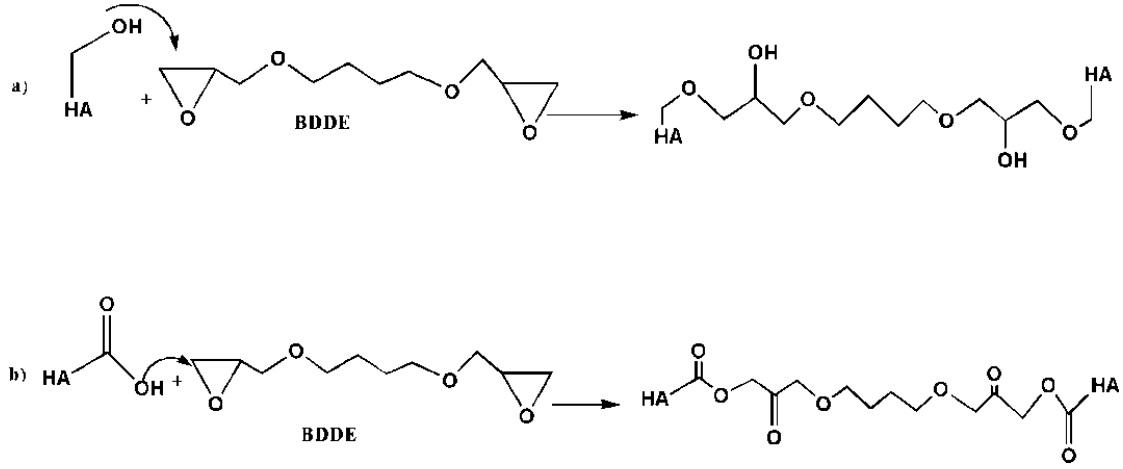


Figure 8 *HA crosslinking with BDDE in alkaline conditions (a), and in acidic conditions (b.). [46]*

2.6 Goal of the work

The main goal of this study is the preparation of water insoluble microfibres from HA, using the method of wet spinning. The fibres are crosslinked with EDC or combination of EDC and NHS crosslinking reagents and the goal of the work also involves the characterization and comparison of prepared fibres and optimization of conditions for preparation of stable fibres.

3 EXPERIMENTAL PART

3.1 Chemicals

- Sodium hyaluronate (1500-1750 kDa) was purchased from Contipro Biotech s.r.o. (CZ)
- *N*-(3-Dimethylaminopropyl)-*N'*-ethylcarbodiimide hydrochloride was obtained from Sigma-Aldrich (Germany)
- *N*-Hydroxysuccinimide was obtained from Sigma-Aldrich (Germany)
- Sodium hydroxide (P.A.) was purchased from Lach-Ner s.r.o. (CZ)
- Methanol (P.A.) was supplied from Lach-Ner s.r.o. (CZ)
- Ethanol (96 %) was purchased from Moravian distillery, Kojetín (CZ)
- Acetic Acid (G.R., 99.8 %) was purchased from Lach-Ner s.r.o.
- Phosphate buffer saline with pH = 7.4, phosphate buffer saline with pH = 3 and phosphate buffer saline with pH = 11 were prepared using KCl (P.A.) obtained from Lachema; NaCl (P.A.) obtained from Lach-Ner s.r.o.; KH_2PO_4 was obtained from Lachema and $HNa_2O_2P \cdot 12\,H_2O$ (P.A), was obtained Sigma-Aldrich. pH was adjusted by HCl (c = 1 mol.dm^{-3}, G.R., 35-38 %) and NaOH (c = 1 mol.dm^{-3}), both were purchased from Lach-Ner s.r.o.

3.2 Equipments

- Thermogravimetric Analysis (TGA Q 500, TA Instruments)
- Stress-Strain Tester (Zwick Z 010, Roell)
- Fourier Transform Infrared Spectroscopy (Tensor 27, FTIR BRUKER)
- D-8 Advance diffractometer (Bruker AXS, Germany)
- Scanning electron microscope (SEM MIRA3, TESCAN)
- pH Meter S2K712 ISFETCOM JAPAN
- Analytical scale Denver Instrument SI-234A
- Magnetic stirrer IKA® RCT basic
- Steel syringe for wet spinning

3.3 Methods

3.3.1 Preparation of Hyaluronan Fibers

Sodium hyaluronate (1500-1750 KDa, 6 g) was dissolved under stirring in 94 g of water with the addition of NaOH (0.64 g) to obtain homogenous, well-flowing viscous solution suitable for spinning. This solution was pressed (wet-spinning technique; Figure 9) through a nozzle with the diameter of 0.4 mm to the coagulation bath having the composition: 600 ml of methanol and 400 ml of acetic acid (98 %). The prepared fibres were left in the coagulation bath for 15 hours, then washed with absolute methanol and dried.

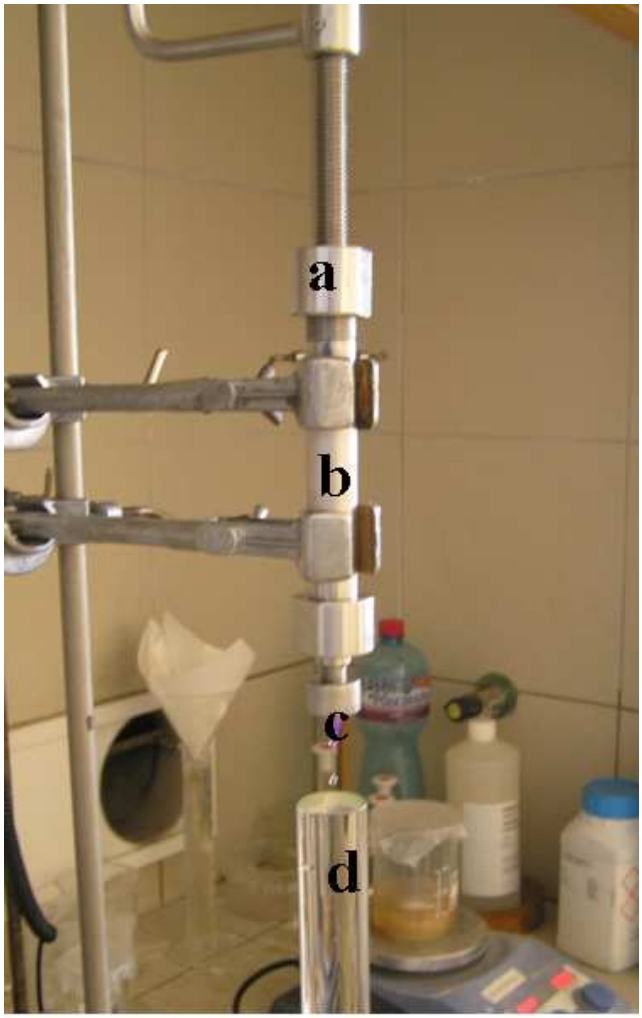


Figure 9 *Photograph of wet-spinning machine; (a) pump part, (b) hyaluronan solution, (c) needle, (d) coagulation bath.*

3.3.2 Preparation of Crosslinking Solution

Different concentrations of EDC were prepared in ethanol (5, 25, 50 and 100 mmol/L) and mixture solutions from EDC and NHS were prepared in ethanol (EDC/NHS, 25/12.5 and 50/25 mmol/L) respectively. The prepared solutions were kept in fridge at 4 °C.

3.3.3 Crosslinking of water soluble hyaluronan fibres with EDC and EDC/NHS

Aqueous mixtures containing an organic solvent (ethanol) of various concentrations of EDC and EDC/NHS were prepared. The water soluble hyaluronan fibres were immersed into the mixtures of the crosslink EDC, EDC/NHS for different time at room temperature. After crosslink with a reaction time of 0.5, 1, 4, 8, 12, 24, 48 h the samples were washed three times with methanol with the change of the solution every 30 min and dried in the air.

3.3.4 Preparation of Samples for Mechanical Tests

For testing mechanical properties of fibres, paper constructions with parameters of 4.5 x 2 cm were prepared, as shown in Figure 10. A single fibre was glued on to the construction, and for each tested type of fibre, three samples were prepared.

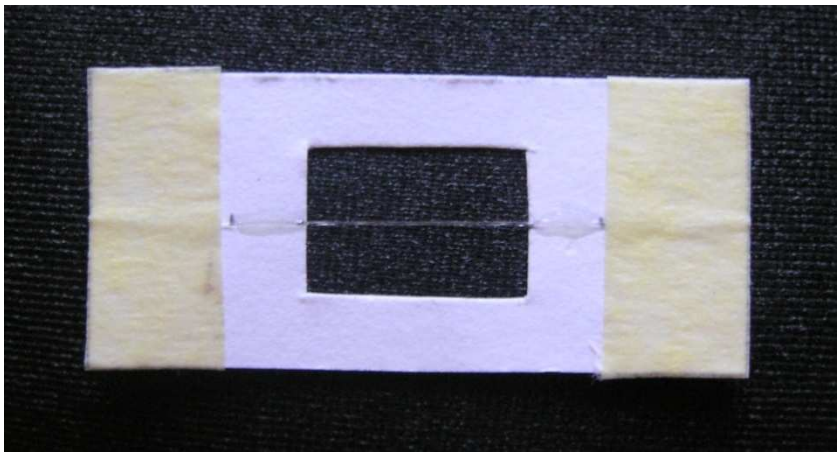


Figure 10 *Paper construction with fibre for mechanical tests.*

3.4 Characterization

3.4.1 Swelling Tests

Swelling tests were performed using analytical scale, in phosphate buffer solution (PBS) at different pHs: 7.4, 3 and 11. The sample of the fibre, prepared as stated in chapter 3.3.3., of approximately 1 cm was weighted with the vial before immersing in PBS solution and after certain times. Every time, the vial was gently dried with small piece of paper before weighing. The swelled fibre with vial was weighted after following times: 1 min, 3 min, 5 min, 10 min, 15 min, 30 min, 45 min, 1 h, 3 h, 5 h, 12 h, 24 h, and 48 h. The swelling ratio was calculated according to equation 1.

$$S(\%) = \frac{w_w - w_d}{w_d} \cdot 100 \quad (1)$$

Where w_w is the weight of wet fibre and w_d is the weight of dry fibre.

3.4.2 Thermal Analysis

Samples were studied using TGA Q 500 (TA Instruments) under heating rate of 10 °C·min^{-1}. The test temperature range was from room temperature to 600 °C with the sample mass of about 5–10 mg under 40 ml·min^{-1} nitrogen atmosphere. The alumina crucible was used for all measurements.

3.4.3 Fourier Transform Infrared Spectroscopy (FTIR)

Attenuated total reflectance Fourier transforms infrared spectroscopy was performed by using a Tensor 27 spectrophotometer (FTIR BRUKER) equipped with a diamond crystal for the ATR-FTIR spectroscopy. The wavenumber range was between 4000 cm^{-1} and 650 cm^{-1} with the resolution of 4 cm^{-1} and the number of scans equal to 32.

3.4.4 Testing of Mechanical Properties

Experimental tests were performed on a Zwick Z 010 testing machine (Roell) with a 500 N force load cell. Tests were performed at a crosshead speed of 5 mm·min^{-1} with jaws for tensile testing, and the pre-load of 0.05 N for 60 s. The samples were in paper construction, as stated in chapter 3.3.4.

3.4.5 Scanning Electron Microscope (SEM)

The images of samples were done at the electron scanning microscope MIRA3 (Tescan). Before recording the photographs, samples were coated with 24 nm film of gold. Accelerating voltage HV 5 kV and detector of secondary electrons were used.

3.4.6 X-Ray Diffraction (XRD)

X-ray diffraction was collected on a device namely; D-8 Advance diffractometer (Bruker AXS) with Bragg-Brentano θ-θ goniometer (radius 217.5 mm) equipped with a secondary beam curved graphite mono-chromator and Na (Tl) I scintillation detector. The generator was operated at 40 kV and 30 mA. The scan was completed at room temperature from 5 to 60° (2θ) in 0.02° step with a counting time of 8 s per step.

4 RESULTS AND DISSCUSSION

EDC and NHS were used for the first time to crosslink water soluble hyaluronan fibres through chemical interaction. Many parameters were studied to optimize the condition of crosslink like concentration of EDC, concentration of NHS, time of reaction. The properties of prepared fibres were studied by chosen types of characterization.

4.1 Characterization by Swelling Tests

The swelling ratio was determined by the swelling tests. The tests were held maximal for 48 hours. The most stable samples were fibres crosslinked for 12, 24 and 48 hours in the solution with concentration of 100 mmol/L EDC. These fibres were stable for 48 hours in pH 7.4, but the less time of crosslinking, the fibre was more fragile and torn into pieces. In ph 3 were all of these three samples equally stable. Comparable to this sample were fibres crosslinked for 48 hours in solutions with concentrations of 50 mmol/L EDC and 50 mmol/L EDC and 25 mmol/L NHS.

In general for all other fibres, the less time of crosslinking and the lower concentration of crosslinking solution, the lower stability they had. In pH 3, the fibres were stable equally or a little longer time than in the solution with pH 7.4.

In pH 11, the samples were stable only for few minutes; they usually hydrolyzed after ten or fifteen minutes. The most stable sample was the fibre crosslinked in the solution of 100 mmol/L for 48 hours, which hydrolyzed after 15 minutes.

In Figure 11 is shown dependence of swelling ratio on time of sample which was crosslinked 48 hours in 100 mmol/L EDC solution. Samples were stable 48 hours in PBS solutions with concentrations 7.4 and 3, in PBS solutions with concentration 11 was fibre stable only 15 minutes.

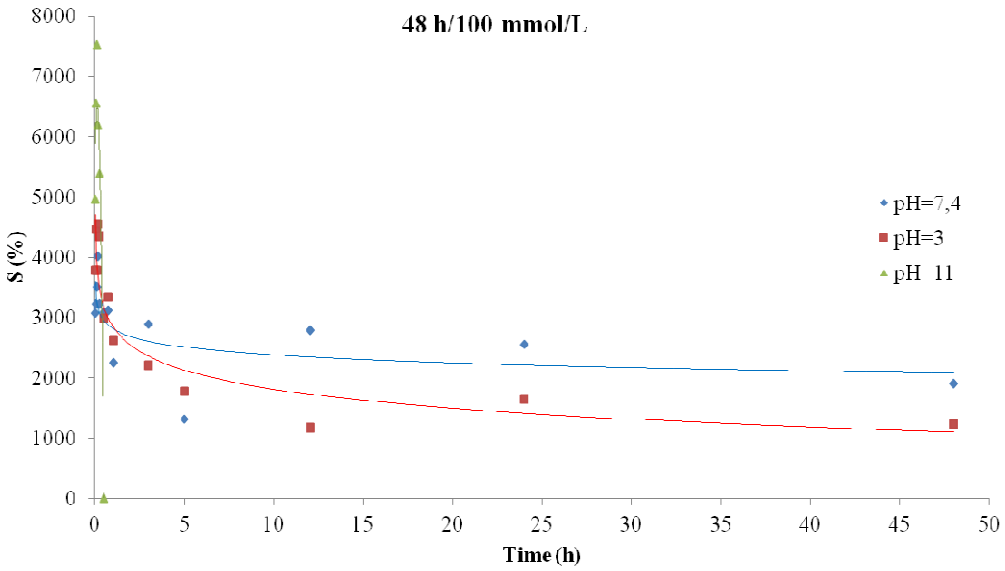


Figure 11 *Result of swelling test for sample crosslinked for 48 h in the solution of 100 mmol/L of EDC.*

4.2 Characterization by Thermogravimetric Analysis

Thermal stability of fibres was studied by TGA Q 500 (TA Instruments). In Figure 12 are displayed TGA curves and theirs derivations of fibre before crosslinking and fibre crosslinked 48 hours in 100 mmol/L EDC solution. The mass loss observed at range from 25 to approximately 190 °C was attributed to the elimination of water either weakly bound to the surface or trapped within the polymer chains and to the elimination of other volatile components (the tracks of coagulation bath or methanol used for washing). At the temperature higher than 185 °C starts the onset of sample degradation, and the second mass loss starting at about 430 °C is due to the degradation of traces of inorganic materials, probably from treatment of fibres. Temperature for the highest rate of change of mass from first derivation for crosslinked fibre (204.96 °C) is slightly higher than the one for fibre before crosslinking (204.05 °C), which means, that the thermal stability has increased.

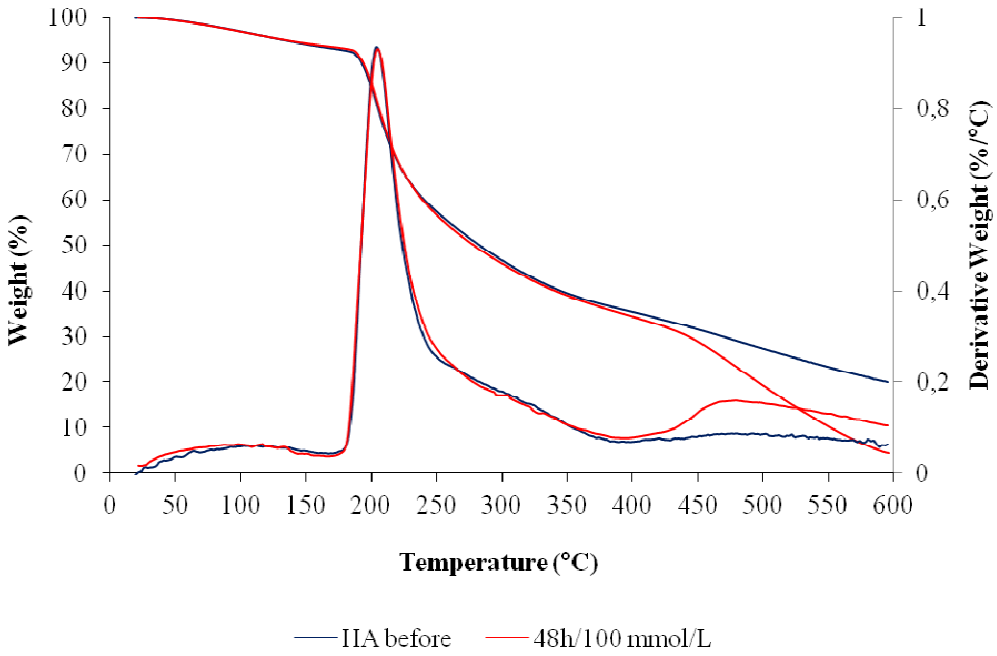


Figure 12 *TGA curve and first derivation of TGA curve of fibre before crosslinking and fibre crosslinked 48 h in 100 mmol/L EDC.*

The determined values of temperatures when the degradation process has started (onset temperatures, T_{ONSET}) and temperatures for the highest rate of change of mass (T_1) obtained through first derivation of TGA curves are given in Table 2.

The results show, that temperatures for the highest rate of change of mass are increasing from fibres crosslinked with the highest concentration of crosslinking reagent to fibres crosslinked with the lowest concentration of reagent. It means, that fibres crosslinked with lower concentration of crosslinking reagent and for shorter time are more thermally stable than samples crosslinked for longer time with higher concentration of reagent. This effect is probably due to the formation of ester bonds, which are in general bonds with low stability, caused by crosslinking reactions. More of these weak ester bonds are formed when higher concentration of reagent is used and when the crosslinking treatment is longer. This could be the reason, why fibres crosslinked with higher concentrations of reagents and for longer time have lower thermal stability than the ones crosslinked with lower concentrations and for shorter time.

Table 2 *Results of thermogravimetrical analysis of chosen samples.*

5 mmol/L EDC					
Time of crosslinking	0 h	1 h	12 h	24 h	48 h
T_{ONSET} (°C)	191.20	195.47	193.97	193.14	194.72
T_1 (°C)	204.05	211.94	210.27	207.21	209.56
25 mmol/L EDC					
Time of crosslinking	0 h	1 h	12 h	24 h	48 h
T_{ONSET} (°C)	191.27	191.98	192.43	194.16	193.33
T_1 (°C)	204.05	206.12	206.94	209.40	208.84
25 mmol/L EDC, 12.5 mmol/L NHS					
Time of crosslinking	0 h	1 h	12 h	24 h	48 h
T_{ONSET} (°C)	191.27	191.77	192.03	193.12	192.45
T_1 (°C)	204.05	205.96	206.27	208.23	206.73
50 mmol/L EDC					
Time of crosslinking	0 h	25 min	24 h	48 h	
T_{ONSET} (°C)	191.27	194.22	194.09	191.36	
T_1 (°C)	204.05	210.83	210.35	205.13	
50 mmol/L EDC, 25 mmol/L NHS					
Time of crosslinking	0 h	25 min	48 h		
T_{ONSET} (°C)	191.27	194.46	191.59		
T_1 (°C)	204.05	210.89	205.25		
100 mmol/L EDC					
Time of crosslinking	0 h	1 h	12 h	24 h	48 h
T_{ONSET} (°C)	191.27	191.75	192.13	191.63	191.68
T_1 (°C)	204.05	205.08	206.10	204.92	204.96

4.3 Characterization by Fourier-transformed Infrared Spectroscopy

The new types of bond linkages were determined using infrared analysis with ATR mode. All the following spectra are normalized.

4.3.1 Powder of Sodium Hyaluronan and Hyaluronan Fibre Before Crosslinking

In Figure 13 are displayed whole spectra of sodium hyaluronate powder and fibre before crosslinking. In general, at 3600-2800 cm^{-1} is the region of CH, NH, and OH

stretching vibrations. The peaks at the end of the spectra are typical for bonds C-O-C, C-C, C-H. However, the region of interest is the area between 1500 and 1800 cm^{-1}. In this area, peaks for esterification and amidation appear.

In Figure 14 is shown section of infrared spectra of sodium hyaluronate powder and fibre before chemical crosslinking. New peak at 1733 cm^{-1} appears. This peak is due to the formation of ester bond between the molecules of HA; probably caused by a so called eternal esterification. This happened after the wet spinning and the coagulation in the mixture of acetic acid/methanol, the ester bonds between disaccharide units of HA are formed. Area at around 1550 cm^{-1} shows that the peak for COO^{2-} (1607 cm^{-1}) has decreased and the peak for amidation has occurred (1630 cm^{-1}). The intensity for peak at 1563 cm^{-1} has also increased, and it is area for amidation too (or for amines respectively).

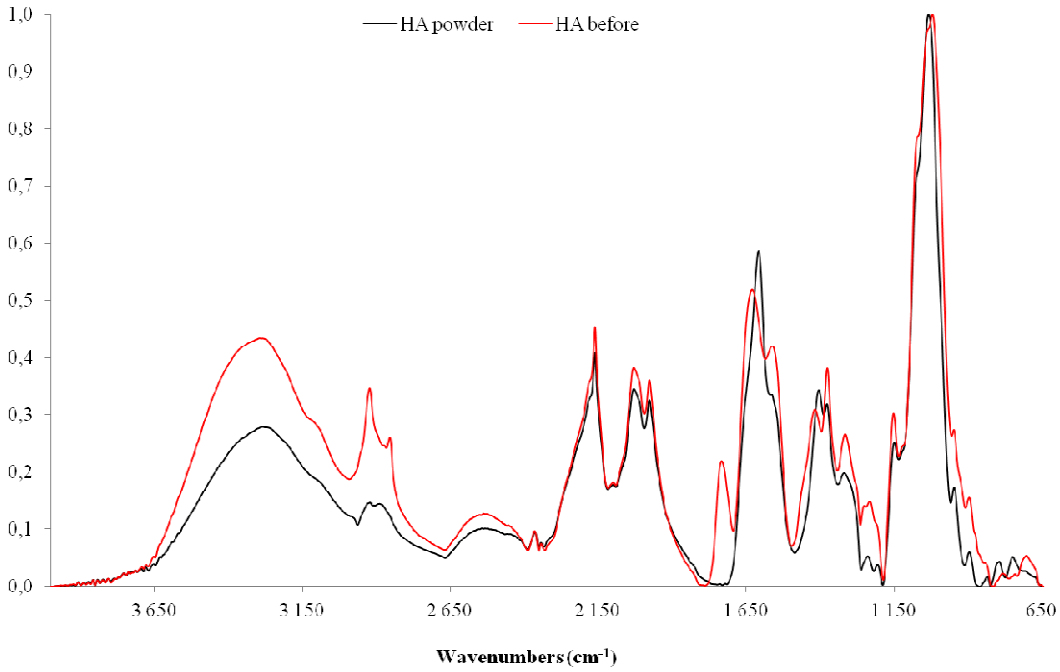


Figure 13 *Complete infrared spectra comparing powder of sodium hyaluronate and hyaluronan fibre before crosslinking.*

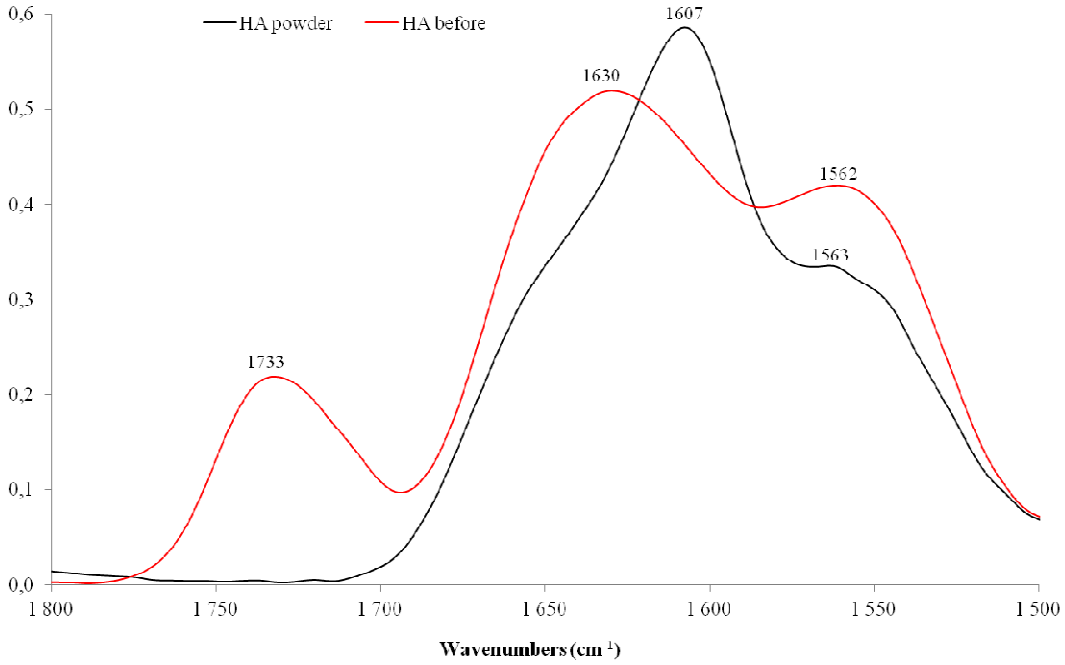


Figure 14 *The section of infrared spectra comparing powder of sodium hyaluronate and hyaluronan fibre before crosslinking.*

4.3.2 Fibres after the treatement with crosslinking reagents

New peak at cca 1700 cm^{-1} is the result of esterification after treatment with EDC (more ester bonds are formed). With the longer time of crosslinking, the peak for ester bond at approximately 1730 cm^{-1} is decreasing and the peak at approximately 1700 cm^{-1} is increasing. This is because of the effect of crosslinking reagent; the longer time of crosslinking, the more ester bonds are formed. This appearance is same for every concentration, however, with the concentration of 25 mmol/L EDC and 25 mmol/L EDC and 12,5 mmol/L NHS, the peak for ester bond shows up after 24 and 48 hours of crosslinking. In the case of samples crosslinked with only the concentration of 5 mmol/L of crosslinking reagent, this peak occurs after 24 hour treatment. The amidation peaks occurred similarly as in case of fibre before crosslinking. The example of this effect is shown in the Figure 15, which is a section from complete infrared spectra of fibre before crosslinking and fibres treated by 100 mmol/L EDC.

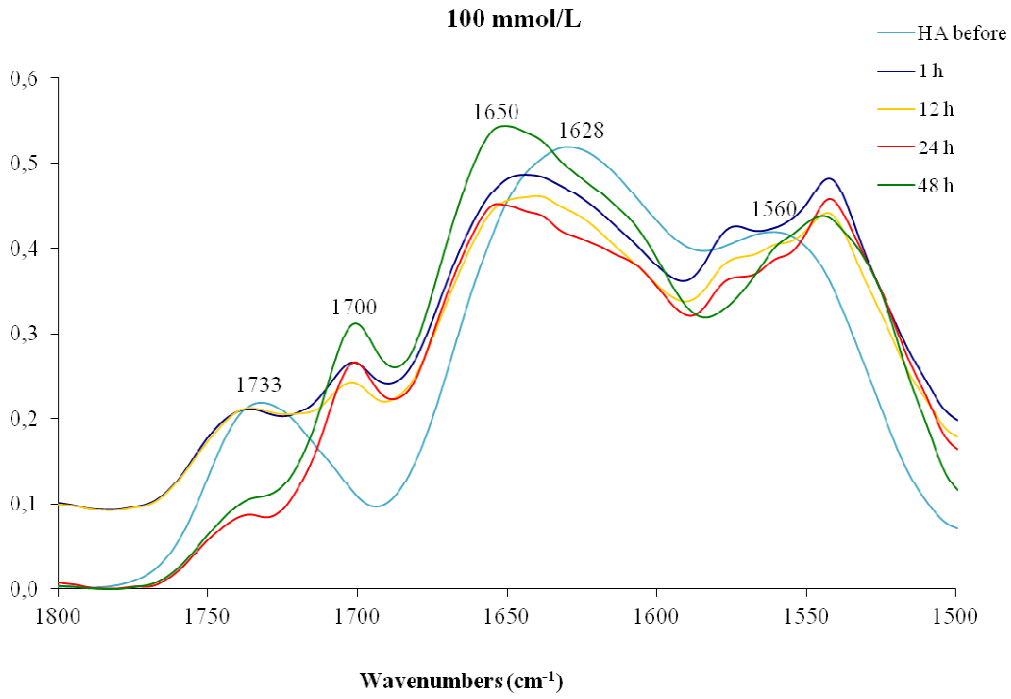


Figure 15 *The section of infrared spectra comparing fibres crosslinked in the solution with concentration of 100 mmol/L EDC and hyaluronan fibre before crosslinking.*

4.4 Characterization of Mechanical Properties

Mechanical properties were characterized by testing machine Zwick Z 010 (Roell). The following values were determined: Youngs modulus, tensile strength and strain at break. The data are, however, very variable, and no dependence is visible. The reason for this behaviour is probably the high heterogeneity of fibres. In the Table 3 are determined values of Youngs modulus, tensile strength and strain at break (the average values of three measurements for each fibre).

Table 3 *Determined values for E-modulus, strength and strain at break.*

Fibre	E-Modulus [GPa]	Strength [MPa]	Strain at Break [%]
HA before	13.15	470.04	13.66
5 mmol/L 1h	9.28	459.09	17.91
5 mmol/L 12h	6.97	367.14	16.59
5 mmol/L 24h	3.16	396.32	19.56
5 mmol/L 48h	7.83	328.11	8.06
25/0 mmol/L 1h	9.44	404.68	14.32
25/0 mmol/L 12h	5.98	501.95	13.66
25/0 mmol/L 24h	5.26	422.27	24.34
25/0 mmol/L 48h	12.58	556.62	11.75
25/12.5 mmol/L 1h	8.31	374.34	12.93
25/12.5 mmol/L 12h	11.37	383.13	8.90
25/12.5 mmol/L 24h	6.88	351.04	10.08
25/12.5 mmol/L 48h	6.90	439.57	14.19
50 mmol/L 25 min	3.60	103.42	12.77
50 mmol/L 48 h	14.12	571.91	12.08
50/25 mmol/L 25 min	4.63	499.53	7.12
50/25 mmol/L 48h	13.40	439.57	8.68
100 mmol/L 1h	4.33	401.28	10.51
100 mmol/L 12h	12.94	445.70	12.11
100 mmol/L 24h	7.16	357.62	18.27
100 mmol/L 48h	11.20	468.31	10.53

In the following Figure 16 are displayed tensile curves of pure hyaluronan fibre. The curves exhibit a linear elastic behaviour up to approximately 0.3 % of strain, consequently shallow yield point is observed and quasi plateau area. The end of the curves is the break point. For example are shown also stress-strain curves of fibre crosslinked 48 hours in 100 mmol/L EDC (Figure 17), where the curves have similar course as the previous sample.

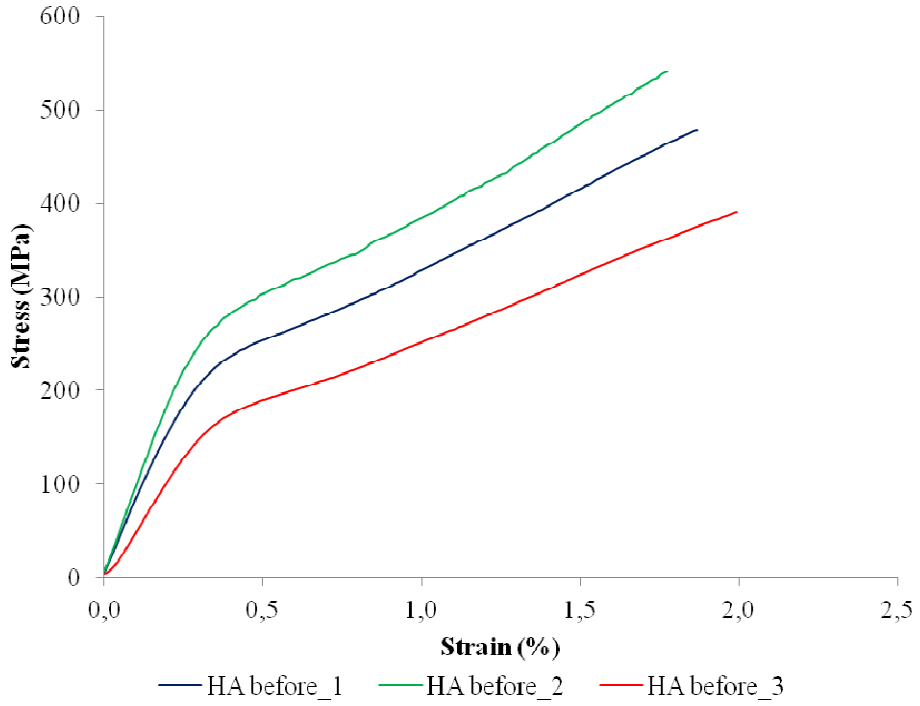


Figure 16 *The stress-strain curves of fibres before crosslinking.*

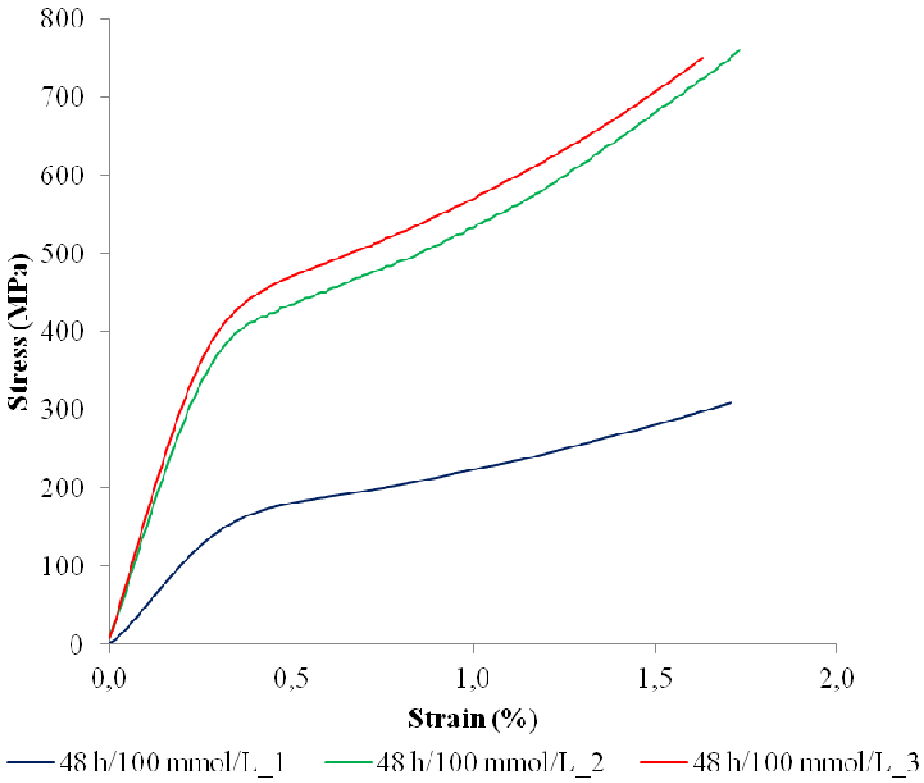


Figure 17 *The stress-strain curves of fibres crosslinked for 48 hours in solution with concentration of 100 mmol/L EDC.*

The determined E-modulus in dependence on concentration of crosslinkin reagent is shown in Figure 18, where are compared fibre before crosslinking and fibres after 48 hours of treatment in solutions of all concentrations. As stated before, no dependence has occurred, probably due to the different diameter of prepared fibres, which influenced the results of mechanical tests.

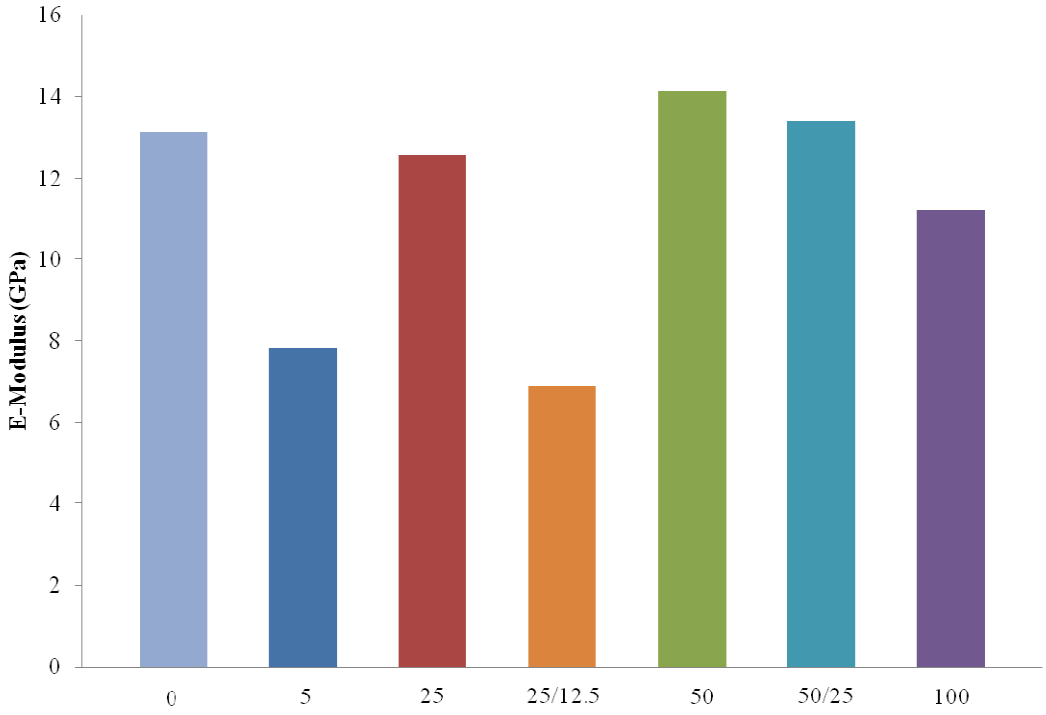


Figure 18 *E-Modulus of sample before crosslinking (0) and samples crosslinked for 48 hours. The concentrations are given under the columns in mmol/L.*

4.5 Characterization by Scanning Electron Microscopy

Figure 19 shows the pictures from scanning electron microscope and cross-section of pure hyaluronan fibres before and after crosslink with EDC and EDC/NHS mixture. Pure hyaluronan fibre before crosslink (A, B) has relatively smooth surface morphology. Hyaluronan fibres after crosslink with EDC without and with NHS at room temperature with concentration of EDC 100 mmol/L, and ratio between EDC and NHS was (2:1) are shown in figures (C, D, E, F) respectively. It is clear from figures (C, E) that hyaluronan fibre after crosslink have similar surface like the fibre before crosslinking. Pictures of cross-sections (B, D, F) prove, that diameter of fibres is not of regular shape, but is very variable.

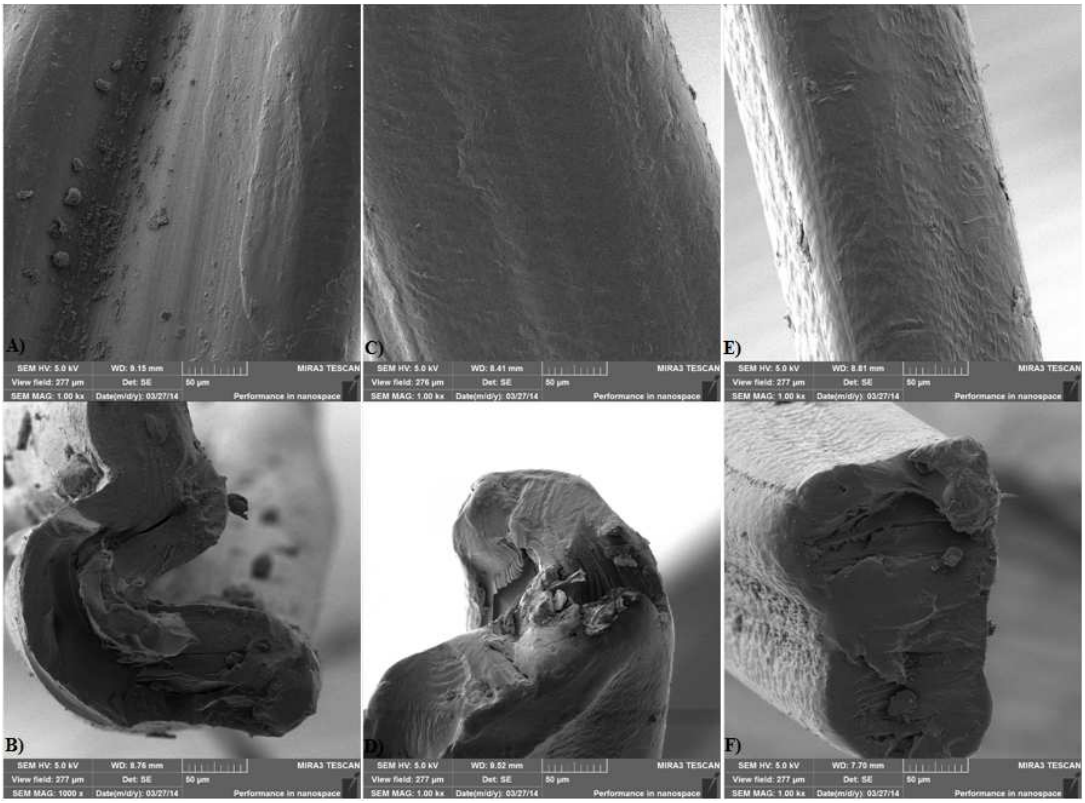


Figure 19 *SEM photographs of surface and cross-section of hyaluronan fibre before crosslinking and after crosslinking. Pure hyaluronan fibre (A), the cross-section of pure hyaluronan fibre (B), hyaluronan fibre crosslinked with 50 mmol/L EDC and 25 mmol/L NHS (C) and the cross-section of this fibre (D), hyaluronan fibre crosslinked with 100 mmol/L EDC (E) and its cross-secion (F).*

4.6 XRD

Figure 20 shows XRD of hyaluronan fibre before crosslinking, and fibres crosslinked for 1 hour in solutions with concentrations 50 mmol/L EDC and 50 mmol/L EDC and 25 mmol/L NHS. Figure 21 shows XRD of hyaluronan fibre before crosslinking, and fibres crosslinked for 24 hours in solutions with concentrations 50 mmol/L EDC and 50 EDC mmol/L and 25 mmol/L NHS. The diffraction pattern of pure hyaluronan fibre shows only broad peak with maximum 2-theta about 20° and second smaller broad peak with maximum 2-theta about 9°, corresponding to amorphous structures. Fibres after treatment for 1 and 24 hours with concentrations of 50 mmol/L EDC exhibit comparable diffraction patterns with two broad peaks at same angle 2-theta without any sharper peaks corresponding to crystallic structure. For fibres treated by 50 mmol/L EDC and 25 mmol/L NHS solution, one sharp peak occurs at angle 2-theta = 18.1° (corresponds to interlayer distance 4.899 Å).

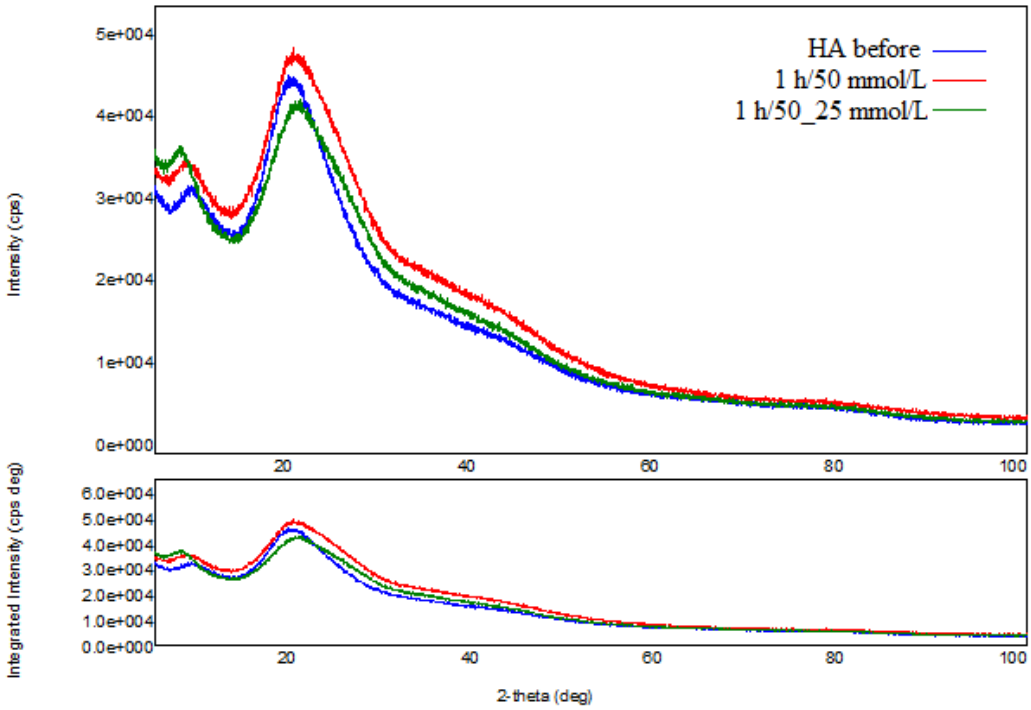


Figure 20 *XRD of fibre before crosslinking, fibres crosslinked for 1 hour in solutions with concentrations of 50 mmol/L EDC and 50 mmol/L EDC with 25 mmol/L NHS.*

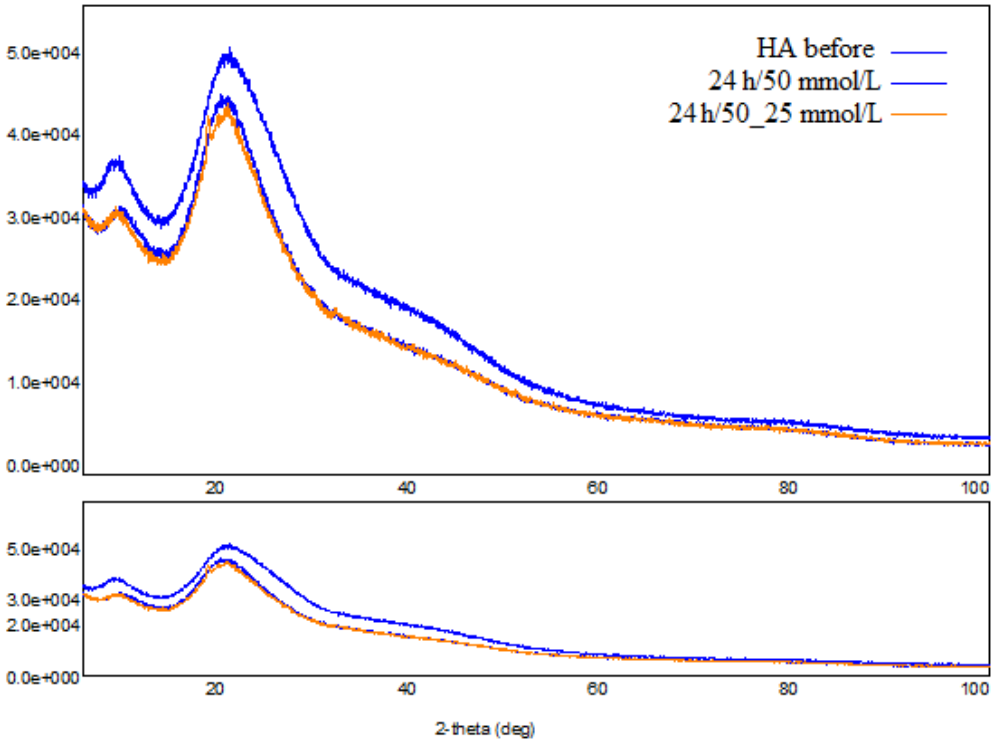


Figure 21 *XRD of fibre before crosslinking, fibres crosslinked for 24 hours in solutions with concentrations of 50 mmol/L EDC and 50 mmol/L EDC with 25 mmol/L NHS.*

5 CONCLUSION

The aim of presented work was to prepare stabile hyaluronan microfibers in water environment and optimization of the conditions. The purpose was also their characterization and comparison before crosslinking and after crosslinking, and between crosslinked fibres mutually.

The fibre before crosslinking is stable in solution with pH 7.4 and 3 only for approximately 10 minutes, in solution with pH 3 for 5 minutes. The FTIR spectra of this sample demonstrated that new peak for esterification has occurred at 1733 cm^{-1}.

When fibers were crosslinked, their stability in all solutions (pH 7.4, 3, 11) has increased. With higher concentration of crosslinking solution and longer time of crosslinking, the fibres were more stable in these solutions.

The dependence: the higher concentration of crosslinking solution and longer time of crosslinking, also holds for new esterification peak which appeared after crosslinking, approximately at 1700 cm^{-1}. Crosslinked fibres have two peaks for ester bonds, first at around 1730 cm^{-1} for esterification after spinning (so called *eternal esterification*), and second one at around 1700 cm^{-1} after the crosslinking with crosslinking reagents.

Thermogravimetrical analysis showed that all the tested crosslinked fibres have higher thermal stability than the fibre before crosslinking.

The mechanical properties of the fibres are highly influenced by the heterogeneous cross-section of the fibres; therefore no dependence has occurred when determining these properties.

As the best sample, was chosen the fibre crosslinked for 48 hours in the solution with concentration of 100 mmol/L of EDC. This fibre was stable in neutral and acidic solution for 48 hours and in alkali solution 15 minutes. Thermogravimetrical analysis determined the rate of change of the mass of this sample as 204.96 °C, which is only a little higher than the same value for fibre before crosslinking (204.05 °C).

In conclusion, more types of crosslinked fibres and not crosslinked fibre were prepared and characterized with different techniques. Advantages of such preparation of fibres are its no toxicity, the fact that the procedure is a green technology because it is no-polluting, and there is no evaporation of the chemical agents during the process. Besides, it is a relatively inexpensive method thanks to the system water and sodium hydroxide. General conclusion is that fibres stable in water were prepared with the crosslinking reagents EDC and NHS. With higher concentration of crosslinking reagent and time of crosslinking, fibres are more stable in PBS solutions and show higher degree of esterification. Considering the mechanical properties, no important dependence was found. It is probably due to the heterogeneity the fibres, and this problem could be the new topic for solving in the future.

6 REFERENCES

[1] Papkov, S. P.: Correlation of natural and man-made polymer fibre structures. Fibre Chemistry. 1997, vol. 29, no. 1.

[2] Tamayol, A.; Akbari, M.; Annabi, N.; et. col.: *Fiber-based tissue engineering: Progress, challenges and opportunities.* Biotechnology Advances. 2013, vol. 31, p. 669-687.

[3] He, N.; Ke, Q.; Huang, C.; et. col.: Needle-punched nonwoven matrix from regenerated collagen fiber for cartilage tissue engineering. Journal of Applied Polymer Science. 2014.

[4] Malafaya, P. B.; Silva, G. A.; Reis, R. L.: Natural-origin polymers as carriers and scaffolds for biomolecules and cell delivery in tissue engineering applications. Advanced Drug Delivery Reviews. 2007, vol. 59, p. 207-233.

[5] Ramakrishna, S.; Mayer, J.;Wintermantel, E.; Leong, K. W.: *Biomedical applications of polymer-composite materials: a review.* Composites Science and Technology. 2001, vol. 61, p. 1189-1224.

[6] Necas, J.; Bartosikova, L.; Brauner, P.; Kolar, J.: *Hyaluronic acid (hyaluronan): a review.* Veterinarni Medicina. 2008, vol. 53, p. 397-411.

[7] Price, R. D.; Berry, MG.; Navsaria, H. A.: *Hyaluronic acid: the scientific and clinical evidence.* Journal of Plastic, Reconstructive and Aesthetic Surgery. 2007, vol. 60, p. 1110-1119.

[8] Abdel-Mohsen, A. M.; Hrdina, R.; Burgert, L.; et. col.: *Antibacterial activity and cell viability of hyaluronan fiber with silver nanoparticles.* Carbohydrate Polymers. 2013, vol. 92, p. 1177-1187.

[9] Perepelkin, K. E.: Structure and structural mechanics of polymer fibres: current concepts. Fibre Chemistry. 2009, vol. 41, no. 1.

[10] Adikwu, M. U.: *Biopolymers in drug delivery. Recent advances and challenges.* Bentham Science Publishers Ltd. 2009. ISBN 978-1-60805-078-9.

[11] Papkov, S. P.: Correlation of natural and man-made polymer fibre structures. Fibre Chemistry. 1997, vol. 29, no. 1.

[12] Pakshver, E. A.: Regulation of the structure of fibres from polymer solutions. Fibre Chemistry. 2006, vol. 38, no 4.

[13] Yudanova, T. N.; Skokova, I. F.; Gal'braikh, L. S.: *Fabrication of biologically active fibre materials with predetermined properties.* Fibre Chemistry. 2000, vol. 32, no. 6.

[14] Huang, C. H.; Lee, M. I.; Kim, S.: An inverse problem in determining the acid and salt diffusivities simultaneously for polymer solution in a wet spinning problem. Applied Mathematical Modeling. 2013, vol. 37, p. 1108-1125.

[15] Gao, Q.; Shen, X.; Lu, X.: *Regenerated bacterial cellulose fibers prepared by the NMMO·H2O process.* Carbohydrate polymers. 2011, vol. 83, p. 1253-1256.

[16] Meyer, M.; Baltzer, H.; Schwikal, K.: *Collagen fibres by thermoplastic and wet spinning.* Materials Science and Engineering C. 2010, vol. 30, p. 1266-1271.

[17] Um, I. C.; Kweon, H. Y.; Lee, K. G.; et. col.: *Wet spinning of silk polymer I. Effect of coagulation conditions on the morphological feature of filament.* International Journal of Biological Macromolecules. 2004, vol. 34, p. 89-105.
[18] Lee, S. H.; Park, S. M.; Kim, Y.: Effect of the concentration of sodium acetate (SA) on crosslinking of chitosan fiber by epichlorohydrin (ECH) in a wet spinning system. Carbohydrate polymers. 2007, vol. 70, p. 53-60.
[19] Lee, K. Y.; Jeong, L.; Kang, Y. O.; et. col.: *Electrospinning of polysaccharides for regenerative medicine.* Advanced Drug Delivery Reviews. 2009, vol. 61, p. 1020-1032.
[20] Miao, Y. E.; Zhu, H.; Chen, D.; et col.: Electrospun fibres of layered double hydroxide/biopolymer nanocomposites as effective drug delivery systems. Materials Chemistry and Physics. 2012, vol. 134, p. 623-630.
[21] Sill, T. J.; Von Recum, H. A.: *Electrospinning: Applications in drug delivery and tissue engineering.* Biomaterials. 2008, vol. 29, p. 1989-2006.
[22] Okutan, N.; Terzi, P.; Altay, F.: Affecting parameters on electrospinning process and characterization of electrospun gelatine nanofibers. Food Hydrocolloids. 2014, vol. 39, p. 19-26.
[23] Chandler, K. W.; Peddieson, J.; Idem, S. A.; Rochelle, S. G.: *An improved 1D fiber dry spinning mass transfer model.* Mechanics research Communications. 2002, vol. 29, p. 351-357.
[24] Gou, Z.; McHugh, A. J.: *Two-dimensional modeling of dry spinning of polymer fibers.* Journal of Non-Newtonian Fluid Mechanics. 2004, vol. 118, p. 121-136.
[25] Sen, A. K.: *Coated textiles. Principles and Applications.* Technomic Publishing Company, Inc. 2001. ISBN 1-58716-023-4.
[26] Lawrence, C. A.: *Fundamentals of spun yarn technology.* CRC Press LLC. 2003. ISBN 1-56676-821-7.
[27] Salamone, J. C.: *Polymeric materials encyclopedia.* CRC Press, Inc. 1996. ISBN 0-8493-24T0-X.
[28] Vogel, R.; Tändler, B.; Häussler, L.; et. col.: Melt Spinning of Poly(3-hydroxybutyrate) Fibers for Tissue Engineering Using α-Cyclodextrin/Polymer Inclusion Complexes as the Nucleation Agent. Macromolecular Bioscience. 2006, vol. 6, p. 730-736.
[29] Van de Velde, K.; Kiekens, P.: Biopolymers: overview of several properties and consequences on their applications. Polymer Testing. 2002, vol. 21, p. 433-442.
[30] Rigby, A.J.; Anand, S.C.; Horrock, A.R.: *Textile Materials for Medical and Healthcare Applications*. J. Text, Inst. 1997, vol. 88, p. 83-93.
[31] Kolander, C.: *In a silk workers notebook.* Interweave press. Inc., Loveland, CO. 1985, p. 1-5.
[32] Chandra, R.; Rustgi, R.: *Biodegradable polymers.* Prog. Polym. Sci. 1998, vol. 23, p. 1273-1335.
[33] Mano, J. F., Silva, G. A., Azevedo, H. S, et. col.: Natural origin biodegradable systems in tissue engineering and regenerative medicine: present status and some moving trends. Journal of the Royal Society. 2007, vol. 4, p. 999-1030.

[34] Dang, J. M.; Leong, K. W.: *Natural polymers for gene delivery and tissue engineering.* Advanced Drug Delivery Reviews. 2006, vol. 58, p. 487-499.
[35] http://www.princeton.edu/~achaney/tmve/wiki100k/docs/Polysaccharide.html
[36] [online]. [cit 2014-02-08].
[37] Scheirs, J.; Long, T. E.: *Chemistry and technology of polyesters and copolyesters.* Antony Rowe Ltd. 2003. ISBN 0-471-49856-4.
[38] Kogan, G., Šoltés, L., Stern, R., Gemeiner, P.: Hyaluronic acid: a natural biopolymer with a broad range of biomedical and industrial applications. Biotechnol Lett. 2007, vol. 29, p. 17-25
[39] Leach, J. B.; Bivens, K. A.; Patrick, Jr.; C. W.; Schmidt, C. E.: *Photocrosslinked Hyaluronic Acid Hydrogels: Natural, Biodegradable Tissue Engineering Scaffolds.* Wiley Periodicals, Inc. 2002.
[40] Necas, J.; Bartosikova, L.; Brauner, P.; Kolar, J.: *Hyaluronic acid (hyaluronan): a review.* Veterinarni Medicina. 2008, vol. 53, p. 397-411.
[41] Collins, M. N., Birkinshaw, C.: *Hyaluronic acid based scaffolds for tissue engineering – A review.* Carbohydrate Polymers. 2013, vol. 92, p. 1262-1279.
[42] Vandamme, E. J., De Baets, S., Steinbüchel, A.: *Biopolymers. Polysacharides I.* Germany. ISBN 3-527-30226-3.
[43] Kučerík, J., Průšová, A., Rotaru, A., et. col.: *DSC study on hyaluronan drying and hydration.* Thermochimica Acta. 2011, vol. 523, p. 245-249.
[44] Průšová, A.; Vergeldt, F. J.; Kučerík, J.: *Influence of water content and drying on the physical structure of native hyaluronan.* Carbohydrate Polymers. 2013, vol. 95, p. 515-521.
[45] Brown, M. B.; Jones, S. A.: Hyaluronic acid: a unique topical vehicle for the localized delivery of drugs to the skin. JEADV. 2005, vol. 19, p. 308-318.
[46] Schanté, C. E.; Zuber, G.; Herlin, C.; Vandamme, T. F.: Chemical modifications of hyaluronic acid for the synthesis of derivatives for a broad range of biomedical applications. Carbohydrate Polymers. 2011, vol. 85, p. 469-489.
[47] Luo, Y., Kirker, K. R., Prestwich, G.D.: *Cross-linked hyaluronic acid hydrogel films: new biomaterials for drug delivery.* Journal of Controlled Release. 2000, vol. 69, p. 169-184.
[48] Shah, M. V., Badle, S. S., Ramachandran, K. B.: Hyaluronic acid production and molecular weight improvement by redirection of carbon flux towards its biosynthesis pathway. Biochemical Engineering Journal. 2013, vol. 80, p. 53-60.
[49] Průšová, A.; Šmejkalová, D.; Chytil, M.; et. col.: *An alternative DSC approach to study hydration of hyaluronan.* Carbohydrate Polymers. 2010, vol. 82, 498-503.
[50] Iannitti, T., Bingöl, A. Ö., Rottigni, V., Palmieri, B.: A new highly viscoelastic hyaluronic acid gel: rheological properties, biocompatibility and clinical investigation in esthetic and restorative surgery. International Journal of Pharmaceutics. 2013, vol. 456, p. 583-592.
[51] Collins, M. N., Birkinshaw, C.: *Physical properties of crosslinked hyaluronic acid hydrogels.* J Mater Sci: Mater Med. 2008, vol. 19, p. 3335-3343.

[52] Segura, T., Anderson, B. C., Chung, P. H., et. col.: *Crosslinked hyaluronic acid hydrogels: a strategy to functionalize and pattern.* Biomaterials. 2005, vol. 26, p. 359-371.
[53] Harris, P. A.; Di Francesco, F.; Barisoni, D.; et. col.: Use of hyaluronic acid and cultured autologous keratinocytes and fibroblasts in extensive burns. The Lancet. 1999, vol. 353.
[54] Luu, H. M. D.; Chen, A.; Isayeva, I. S.: Comparative stability of the bioresorbable ferric crosslinked hyaluronic acid adhesion prevention solutions. Society for Biomaterials. 2013.
[55] Jin, Y. J.; Ubonvan, T.; Kim, D. D.: *Hyaluronic Acid in Drug Delivery Systems.* Journal of Pharmaceutical Investigation. 2010, vol. 40, p. 33-43.
[56] Mayol, L.; Biondi, M.; Russo, L.; et. col.: Amphiphilic hyaluronic acid derivates toward the design of micelles for the sustained delivery of hydrophobic drugs. Carbohydrate Polymers. 2014, vol. 102, p. 110-116.
[57] Lai, J. Y.; Tu, I. H.: Adhesion, phenotypic expression, and biosynthetic capacity of corneal keratocytes on surfaces coated with hyaluronic acid of different molecular weights. Acta Biomaterialia. 2012, vol. 8, p. 1068-1079.
[58] Güven, O.: *Crosslinking and Scission in Polymers.* Kluwer Academic Publishers. 1990. ISBN 0-7923-0547-7.
[59] Tillet, G.; Boutevin, B.; Ameduri, B.: Chemical reactions of polymer crosslinking and post-crosslinking at room and medium temperature. Progress in Polymer Science. 2011, vol. 36, p. 191-217.
[60] Williams, D. L.; Mann, B. K.: *A Crosslinked HA-Based Hydrogel Ameliorates Dry Eye Symptoms in Dogs.* International Journal of Biomaterials. 2013.
[61] Tan, H.; Marra, K. G.: Injectable, Biodegradable Hydrogels for Tissue Engineering Applications. Materials. 2010, vol. 3, p. 1746-1767.
[62] Hemaprabha, E.: *Chemical crosslinking of proteins: a review.* Journal of Pharmaceutical and Scientific Innovation. 2012.
[63] http://en.wikibooks.org/wiki/Structural_Biochemistry/Crosslinking_Technique#cite_note-three-3 [online]. 2012 [cit 2014-01-06].
[64] Renier, D.; Crescenzi, V.; Francescangeli, A.: *New cross-linked derivates of hyaluronic acid.* Canadian patent application. 2002.
[65] Kennedy, J. F.; Phillips, G. O.; Williams, P. A.: *Hyaluronan. Volume 1. Chemical, Biochemical and Biological Aspects.* Woodhead Publishing Limited. 2002. ISBN 1 85573 570 9.
[66] Collins, M. N.; Birkinshaw, C.: Comparison of the Effectiveness of Four Different Crosslinking Agents with Hyaluronic Acid Hydrogel Films for Tissue-Culture Applications. 2007.
[67] Collins, M. N.; Birkinshaw, C.: Morphology of Crosslinked Hyaluronic Acid Porous Hydrogels. 2010.
[68] Hwang, H. D.; Cho, H. J.; Balakrishnan, P.; et. col.: *Cross-linked hyaluronic acid-based flexible cell delivery system: Application for chondrogenic differentiation.* Colloids and Surfaces B: Biointerfaces. 2012, vol. 91, 106-113.

[69] Petersen, S.; Kaule, S.; Teske, M.; et. col.: Development and In Vitro Characterization of Hyaluronic Acid-Based Coatings for Implant-Associated Local Drug Delivery Systems. Journal of Chemistry. 2013.
[70] Pauliukaite, R.; Ghica, M. E.; Fatibello-Filho, O.; Brett, C. M. A.: Electrochemical impedance studies of chitosan-modified electrodes for application in electrochemical sensors and biosensors. Electrochimica Acta. 2010, vol. 55, p. 6239-6247.
[71] Fleury, G.; Schlatter, G.; Brochon, C,; Hadziioannou, G.: From high molecular weight precursor polyrotaxanes to supramolecular sliding networks. The 'sliding gels'. Polymer. 2005, vol. 46, p. 8494-8501.
[72] Koukiotis, C. G.; Karabela, M. M.; Sideridou, I. D.: Mechanical properties of films of latexes based on copolymers BA/MMA/DAAM and BA/MMA/VEOVA-10/DAAM and the corresponding self-crosslinked copolymers using the adipic dihydrazide as crosslinking agent. Progress in Organic Coatings. 2012, vol. 75, p. 106-115.
[73] Kenne, L.; Gohil, S.; Nilsson, E. M.; Karlsson, A.; et. col.: *Modification and-cross-linking parameters in hyaluronic acid hydrogels-Definitions and analytical methods.* Carbohydrate Polymers. 2013, vol. 91, p. 410-418.
[74] Park, J. K.; Yeom, J.; Oh, E. J.; et. col.: Guided bone regeneration by poly(lactic-co-glycolic acid) grafted hyaluronic acid bi-layer films for periodontal barrier applications. Acta Biomaterialia. 2009, vol. 5, p. 3394-3403.
[75] Slusarewicz, P.; Zhu, K.; Hedman, T.: Kinetic characterization and comparison of various protein crosslinking reagents for matrix modification. J Mater Sci: Mated Med. 2010, vol. 21, p. 1175-1181.
[76] Lu, P. L.; Lai, J. Y.; MA, D. H. K.; Hsiue, G. H.: Carbodiimide cross-linked hyaluronic acid hydrogels as cell sheet delivery vehicles: characterization and interaction with corneal endothelial cells. J. Biomater. Sci. Polymer Edn. 2008, vol. 19, p. 1-18.
[77] http://www.piercenet.com/method/carbodiimide-crosslinker-chemistry [online]. 2014 [cit 2014-01-04].
[78] Lai, J. Y.: Solvent Composition is Critical for Carbodiimide Cross-Linking of Hyaluronic Acid as an Ophthalmic Biomaterial. Materials. 2012, vol. 5, p. 1986-2002.
[79] Mädler, S.; Bih, C.; Touboul, D.; Zenobi, R.: *Chemical cross-linking with NHS esthers: a systematic study on amino acid reactivities.* Journal of Mass Spectrometry. 2009, vol. 44. P. 694-706.
[80] Luo, Y.; Prestwich, G. D.: Hyaluronic Acid-N-hydroxysuccinimide: A Useful Intermediate for Bioconjugation. Bioconjugate Chem. 2001, vol. 12, p. 1085-1088.
[81] Tomihata, K.; Ikada, Y.: *Crosslinking of Hyaluronic Acid with Glutaraldehyde.* Journal of Polymer Science. 1997, vol. 35, p. 3553-3559.
[82] Adekogbe, I.; Ghanem, A.: Fabrication and characterization of DTBP-crosslinked chitosan scaffolds for skin tissue engineering. Biomaterials. 2005, vol. 26, p. 7241-7250.

[83] Ramamurthi, A.; Vesely, I.: Evaluation of the matrix-synthesis potential of crosslinked hyaluronan gels for tissue engineering of aortic heart valves. Biomaterials. 2005, vol. 26, p. 999-1010.
[84] Hermanson, G. T.: *Bioconjugate Techniques.* 2008. ISBN 978-0-12-382239-0.
[85] Prestwich, G. D.; Marecak, D. M.; Marecek, J. F.; et. col.: Controlled chemical modification of hyaluronic acid: synthesis, applications, and biodegradation of hydrazide derivates. Journal of Controlled Release. 1998, vol. 53, p. 93-103.
[86] Oh, J. K.; Drumright, R.; Siegwart, D. J.; Matyjaszewski, K.: *The development of microgels/nanogels for drug delivery systems.* Prog. Polym. Sci. 2008, vol. 33, p. 448-477.
[87] Zawko, S. A.; Suri, S.; Truong, Q.; Schmidt, C. E.: *Photopatterned anisotropic swelling of dual-crosslinked hxaluronic acid hydrogels.* Acta Biomaterialia. 2009, vol. 5, p. 14-22.
[88] Roig-Roig, F.; Solans, C.; Esquena, J.; García-Celma, M. J.: Preparation, Characterization, and Release Properties of Hydrogels Based on Hyaluronan for Pharmaceutical and Biomedical Use. Journal of Applied Polymer Science. 2013.

7 APPENDIXES

7.1 The List of Abbreviations

BDDE	butanediol-diglycidyl ether
DVS	divinyl sulfone
ECM	extracellular matrix
EDC	1-Ethyl-3-(3-dimethylaminopropyl) carbodiimide
FTIR	Fourier transform infrared spectroscopy
GAG	glycosaminoglycans
GTA	glutaraldehyde
HA	hyaluronic acid
NHS	*N*-hydroxysuccinimide
PBS	Phosphate buffer saline
PEGDG	poly(ethyleneglycol) diglycidyl ether
SEM	scanning electron microscope
TGA	thermogravimetric analysis

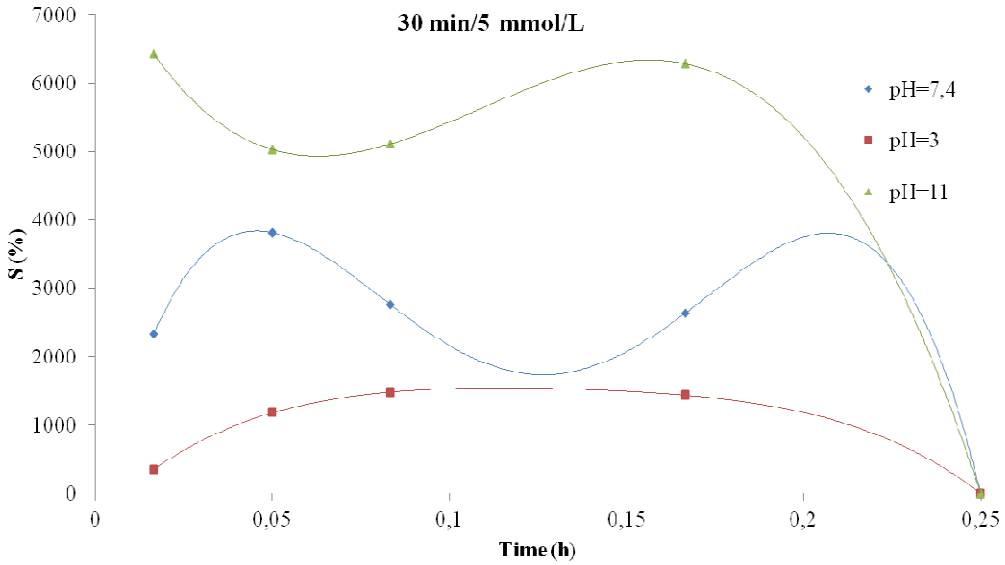
7.2 Swelling Tests

7.3 Thermogravimetric Analysis

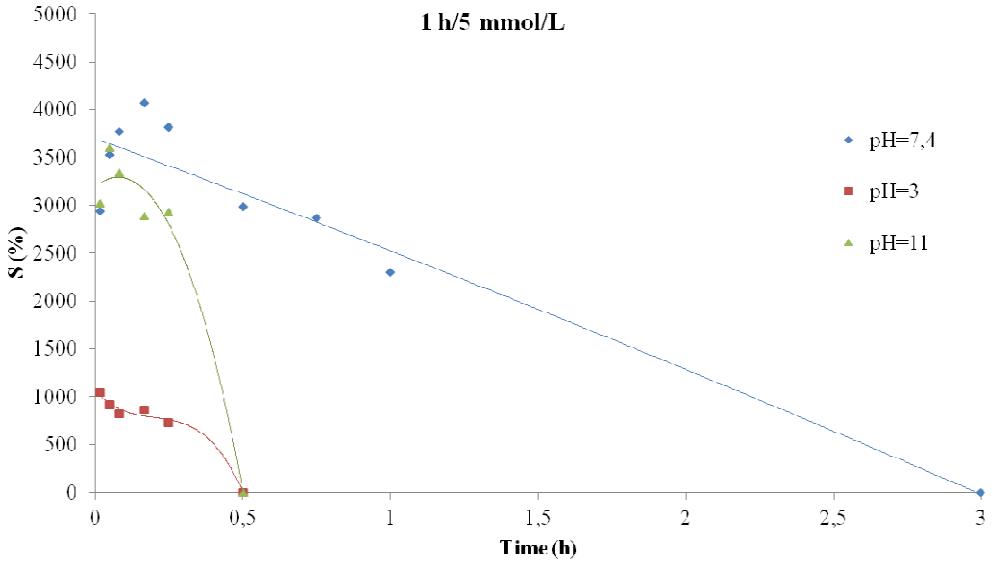
7.4 Infrared Spectroscopy

7.5 Mechnical Testing

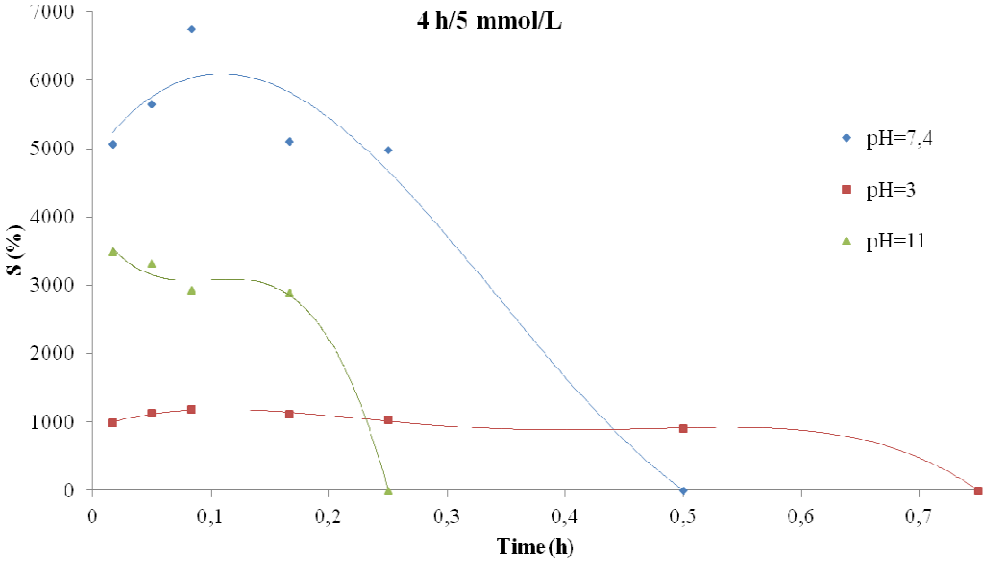
APPENDIX



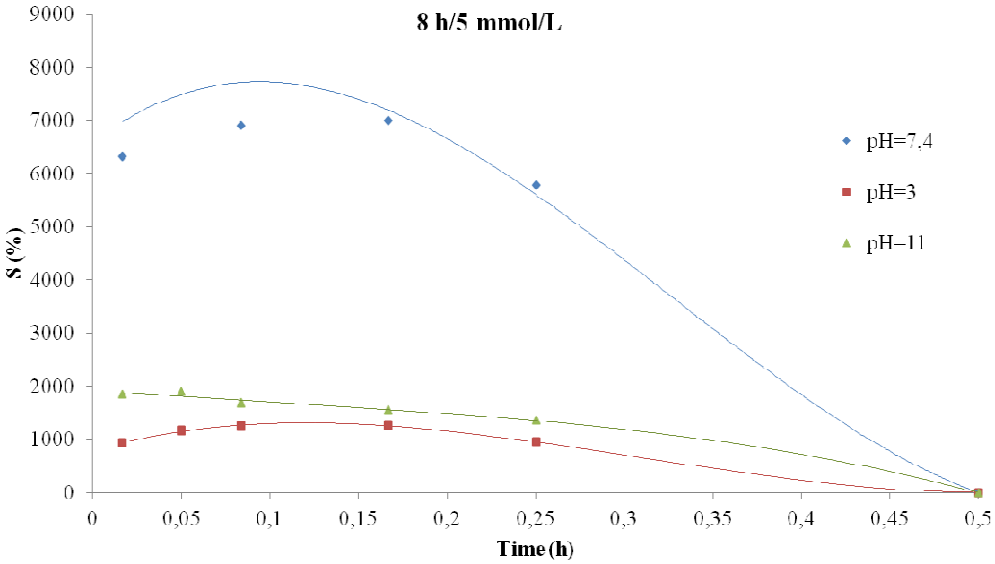
A 1 *Result of swelling test for sample crosslinked for 30 min in the solution of 5 mmol/L of EDC.*



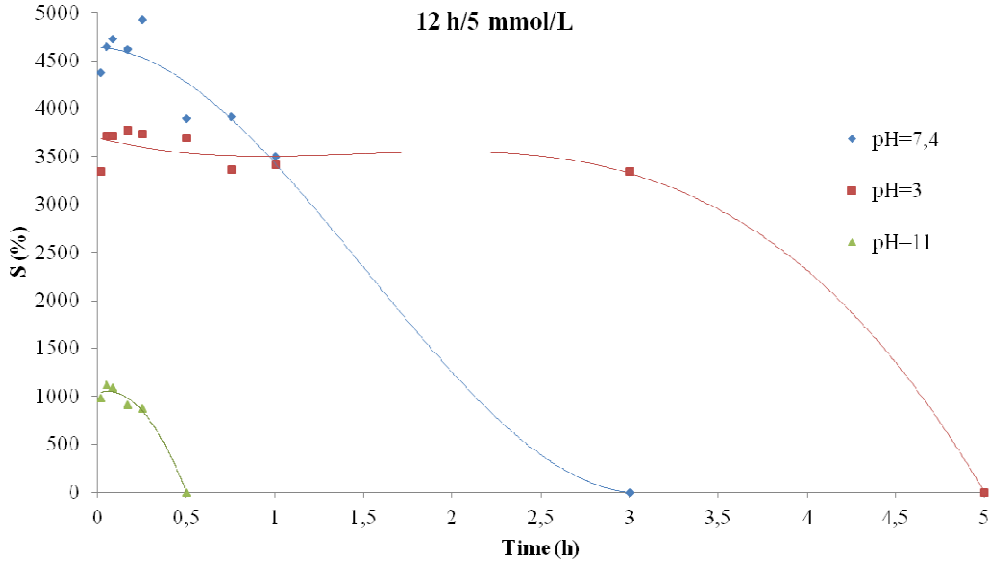
A 2 *Result of swelling test for sample crosslinked for 1 h in the solution of 5 mmol/L of EDC.*



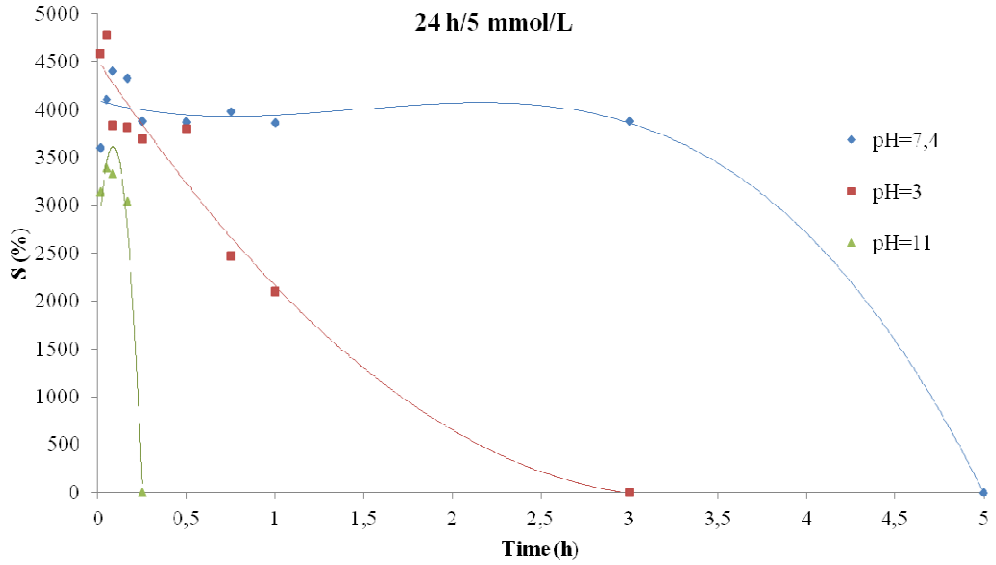
A 3 *Result of swelling test for sample crosslinked for 4 h in the solution of 5 mmol/L of EDC.*



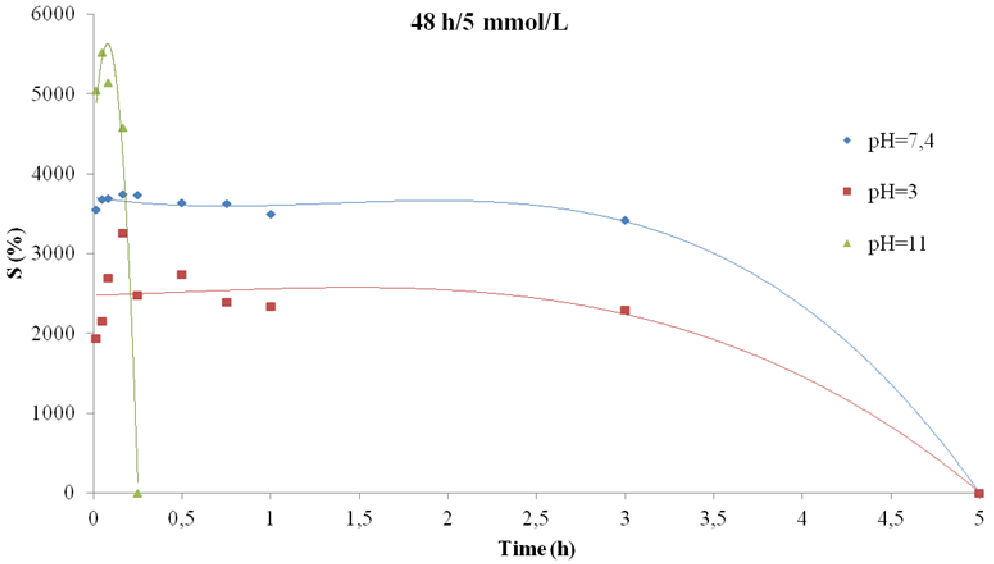
A 4 *Result of swelling test for sample crosslinked for 8 h in the solution of 5 mmol/L of EDC.*



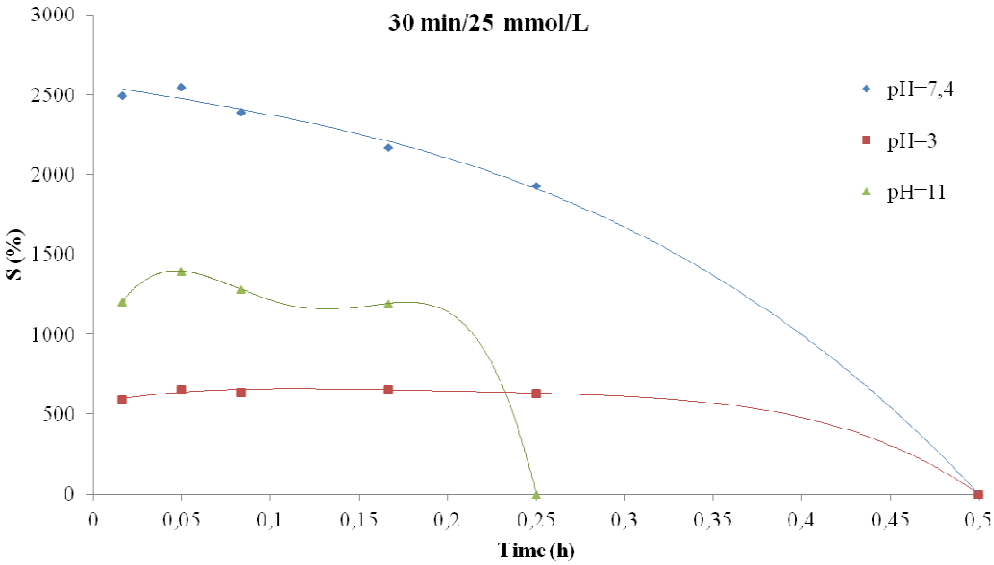
A 5 *Result of swelling test for sample crosslinked for 12 h in the solution of 5 mmol/L of EDC.*



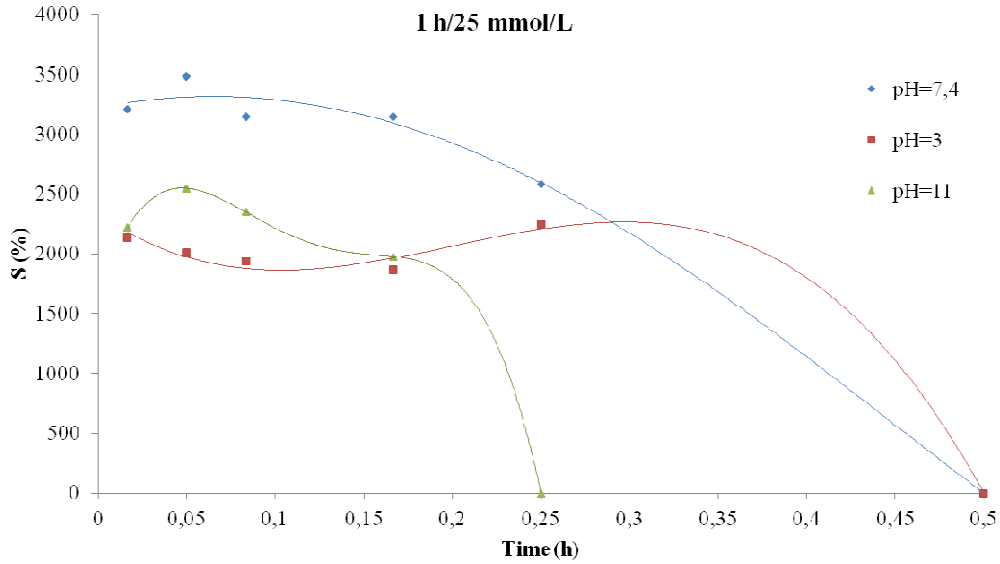
A 6 *Result of swelling test for sample crosslinked for 24 h in the solution of 5 mmol/L of EDC.*



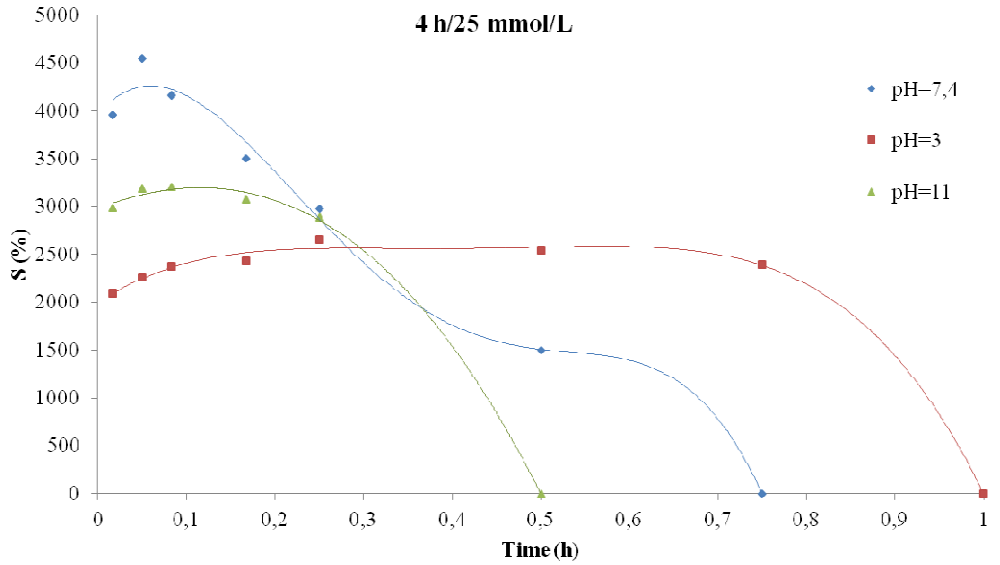
A 7 *Result of swelling test for sample crosslinked for 48 h in the solution of 5 mmol/L of EDC.*



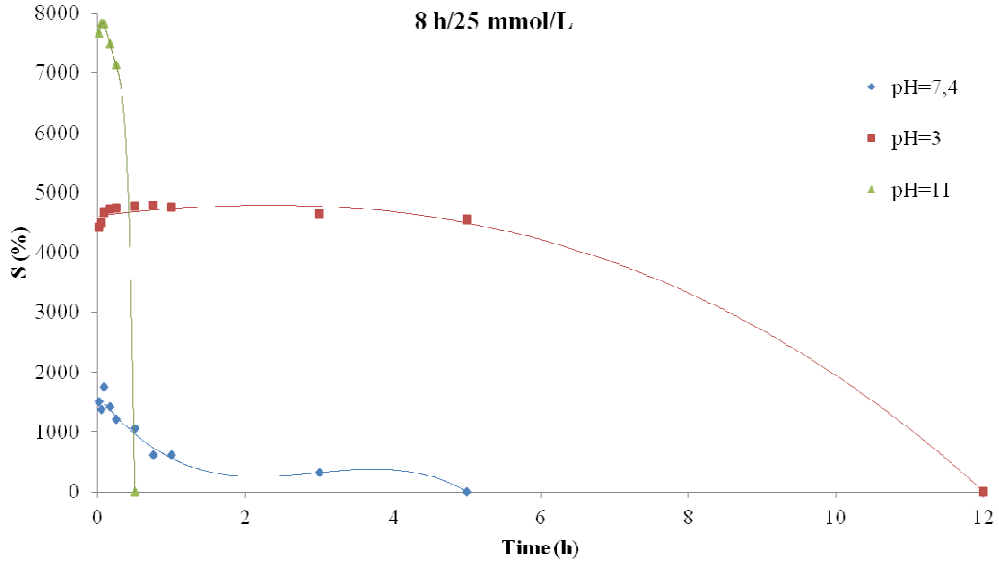
A 8 *Result of swelling test for sample crosslinked for 30 min in the solution of 25 mmol/L of EDC.*



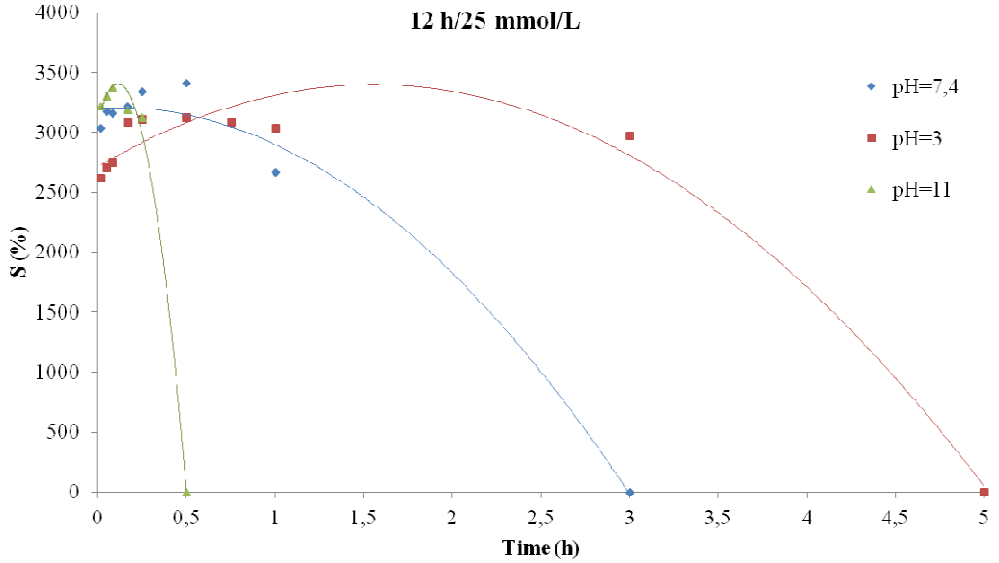
A 9 *Result of swelling test for sample crosslinked for 1 h in the solution of 25 mmol/L of EDC.*



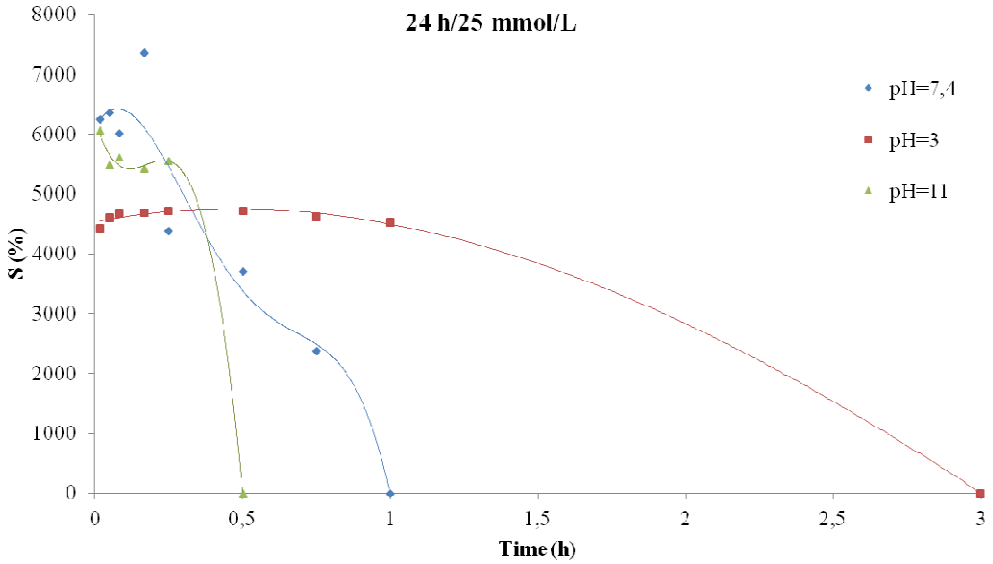
A 10 *Result of swelling test for sample crosslinked for 4 h in the solution of 25 mmol/L of EDC.*



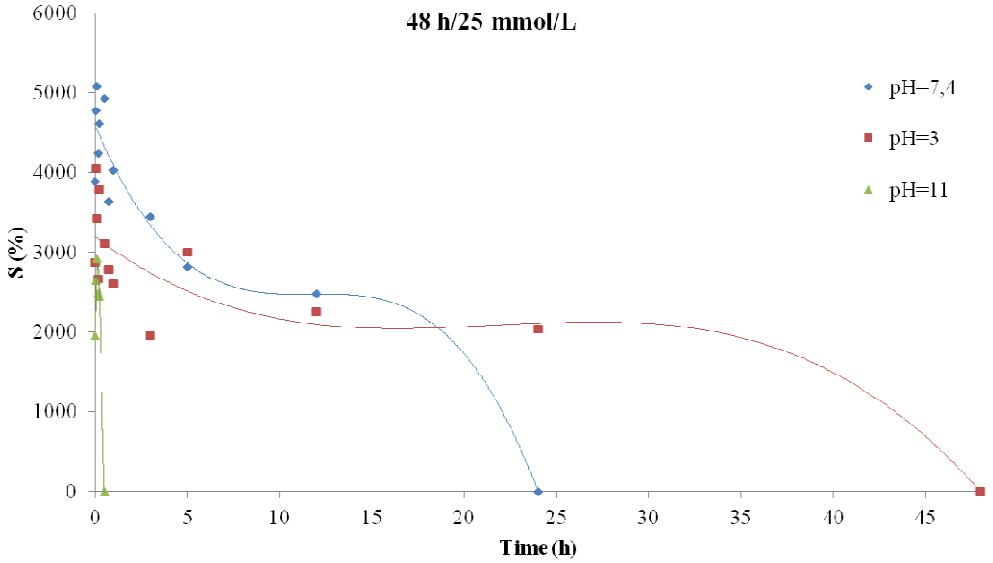
A 11 *Result of swelling test for sample crosslinked for 8 h in the solution of 25 mmol/L of EDC.*



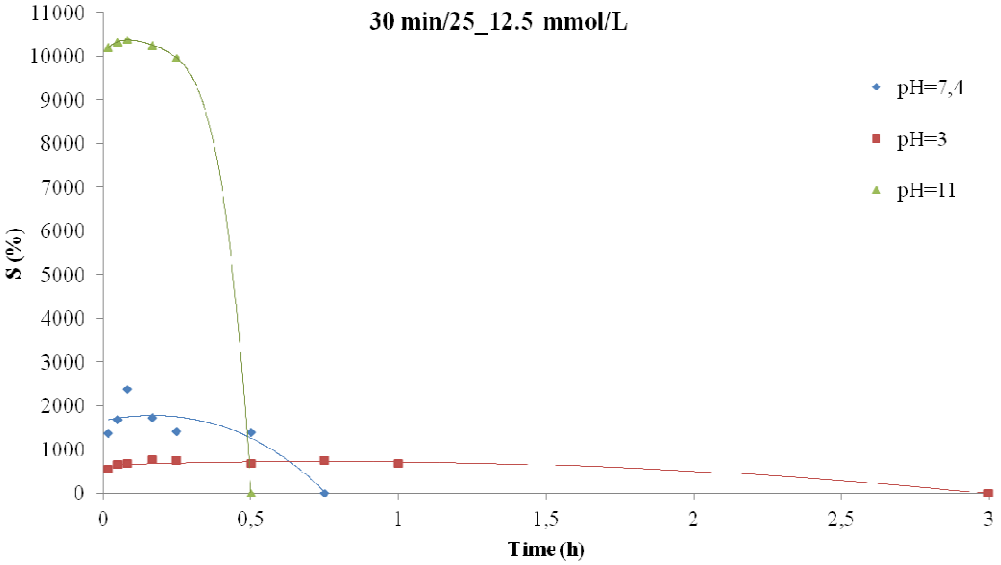
A 12 *Result of swelling test for sample crosslinked for 12 h in the solution of 25 mmol/L of EDC.*



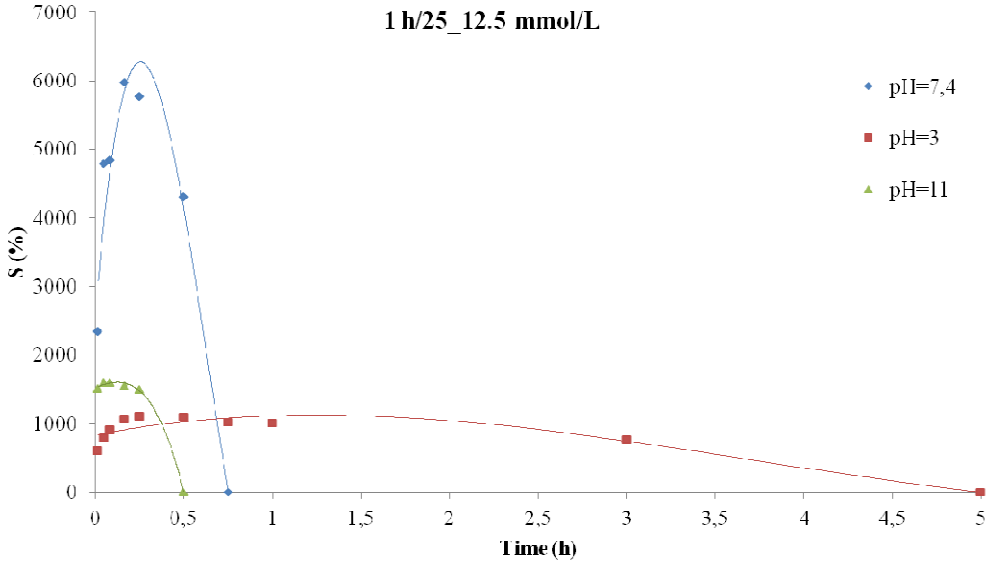
A 13 *Result of swelling test for sample crosslinked for 24 h in the solution of 25 mmol/L of EDC.*



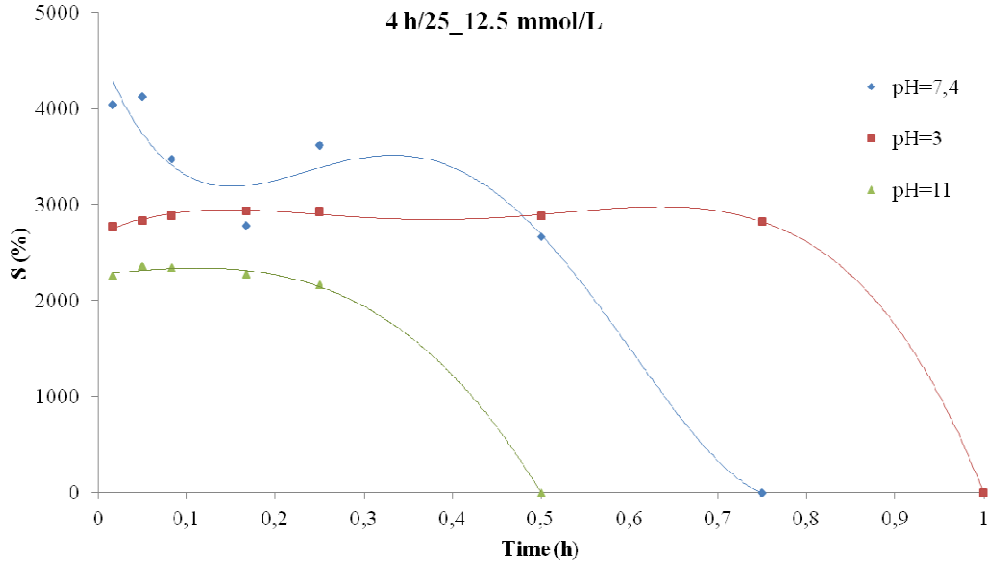
A 14 *Result of swelling test for sample crosslinked for 48 h in the solution of 25 mmol/L of EDC.*



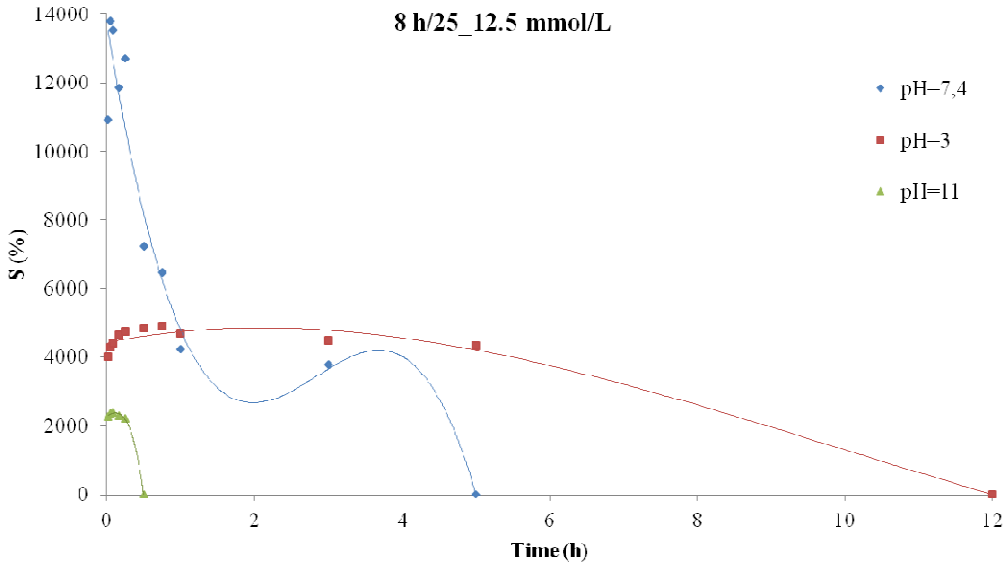
A 15 *Result of swelling test for sample crosslinked for 30 min in the solution of 25 mmol/L of EDC and 12.5 mmol/L of NHS.*



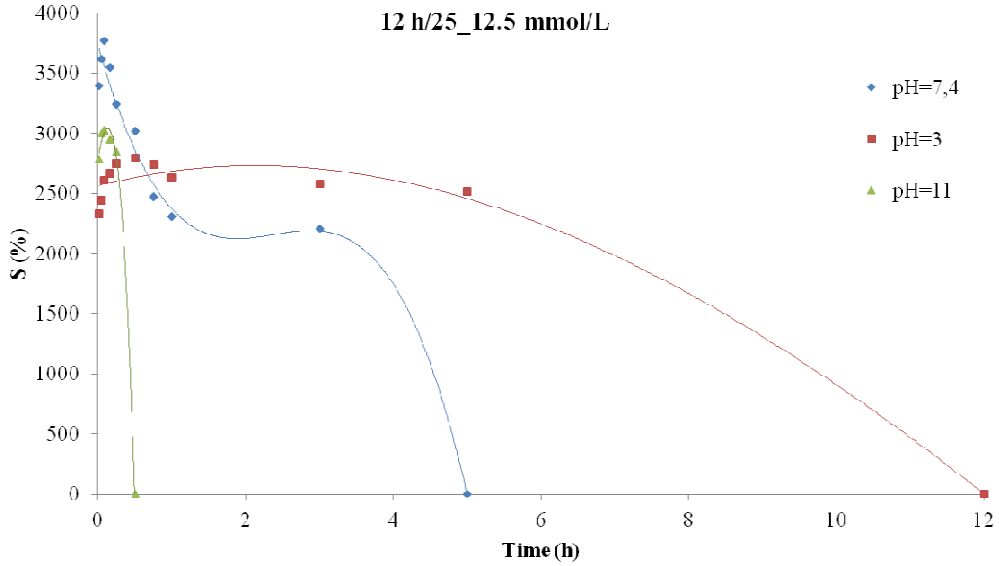
A 16 *Result of swelling test for sample crosslinked for 1 h in the solution of 25 mmol/L of EDC and 12.5 mmol/L of NHS.*



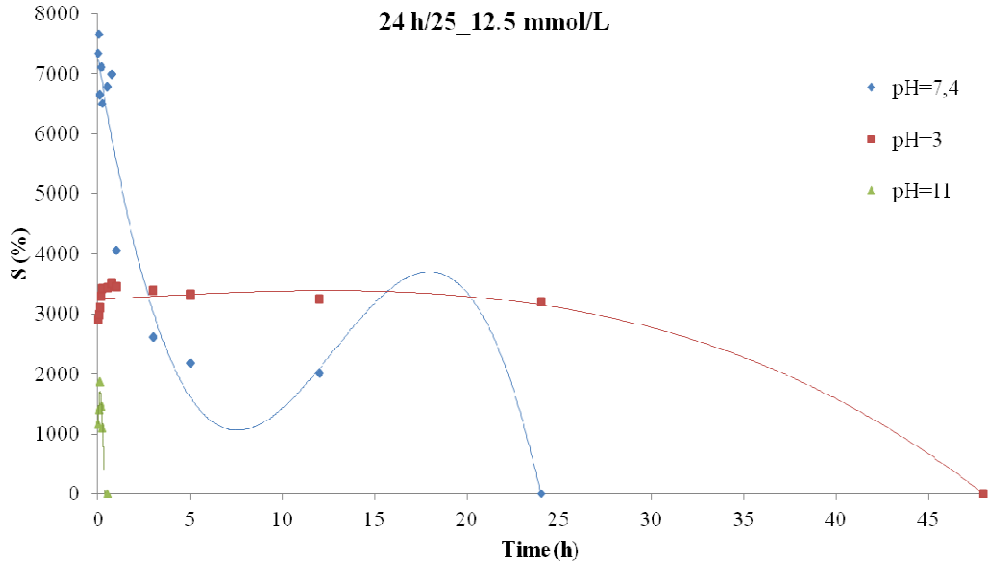
A 17 *Result of swelling test for sample crosslinked for 4 h in the solution of 25 mmol/L of EDC and 12.5 mmol/L of NHS.*



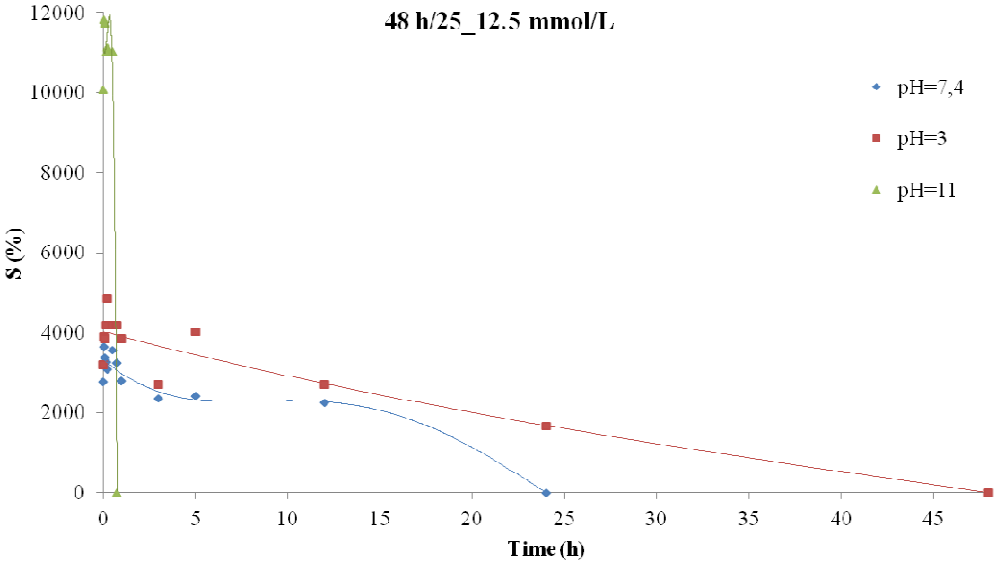
A 18 *Result of swelling test for sample crosslinked for 8 h in the solution of 25 mmol/L of EDC and 12.5 mmol/L of NHS.*



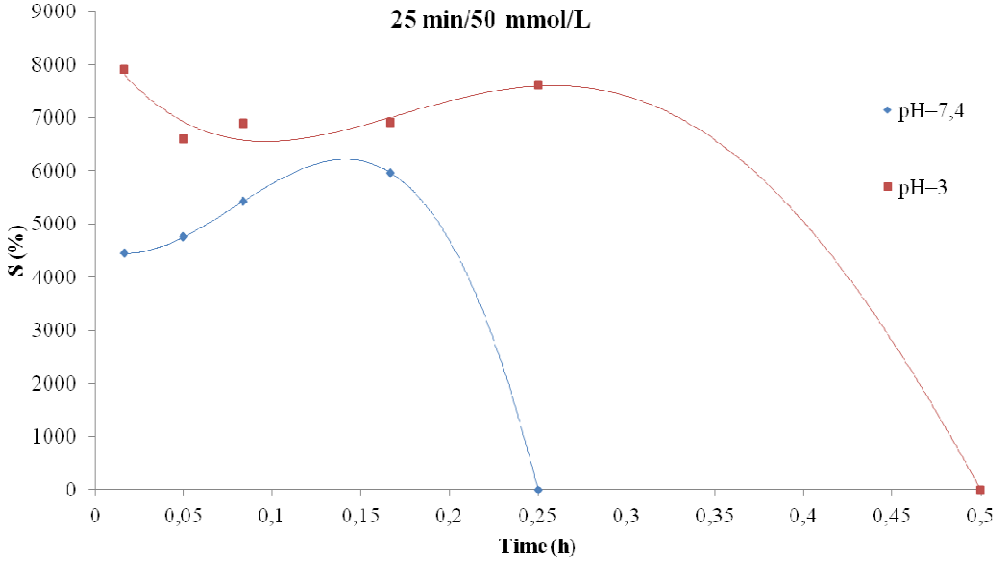
A 19 *Result of swelling test for sample crosslinked for 12 h in the solution of 25 mmol/L of EDC and 12.5 mmol/L of NHS.*



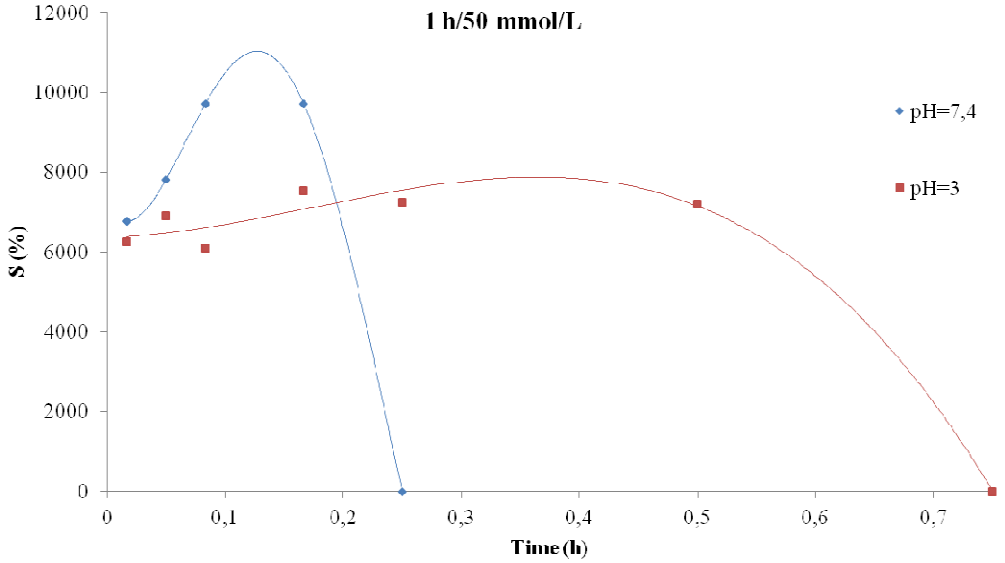
A 20 *Result of swelling test for sample crosslinked for 24 h in the solution of 25 mmol/L of EDC and 12.5 mmol/L of NHS.*



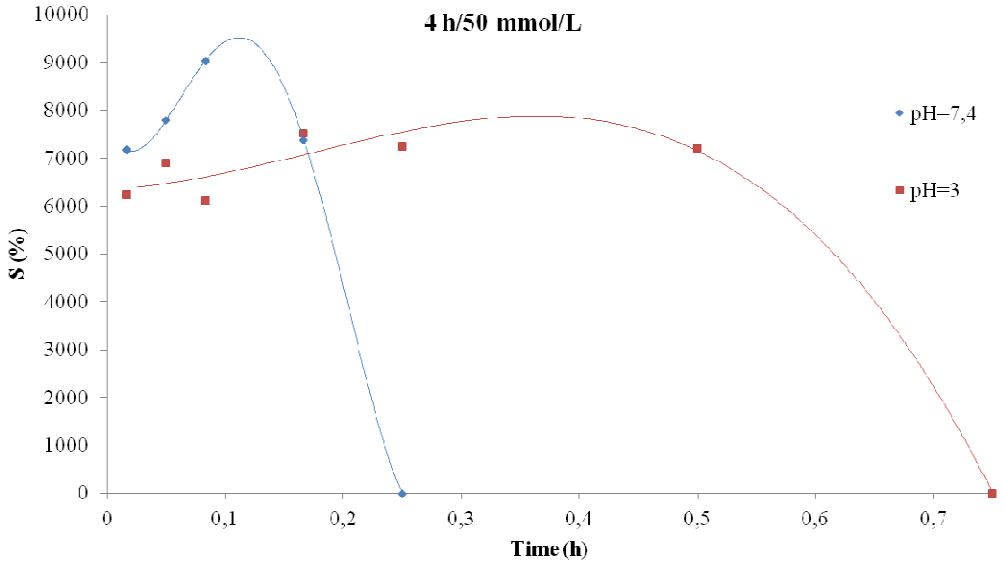
A 21 *Result of swelling test for sample crosslinked for 48 h in the solution of 25 mmol/L of EDC and 12.5 mmol/L of NHS.*



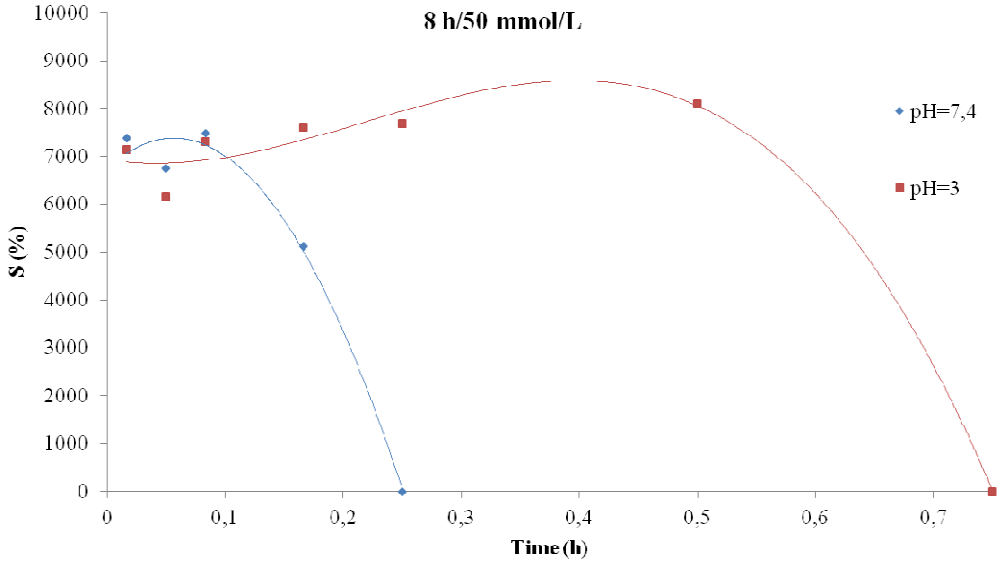
A 22 *Result of swelling test for sample crosslinked for 25 min in the solution of 50 mmol/L of EDC.*



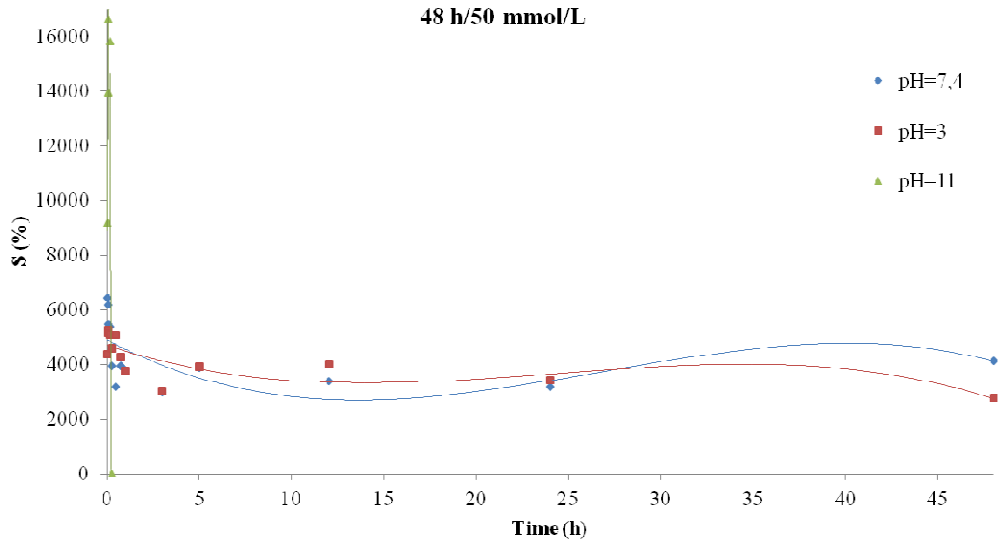
A 23 *Result of swelling test for sample crosslinked for 1 h in the solution of 50 mmol/L of EDC.*



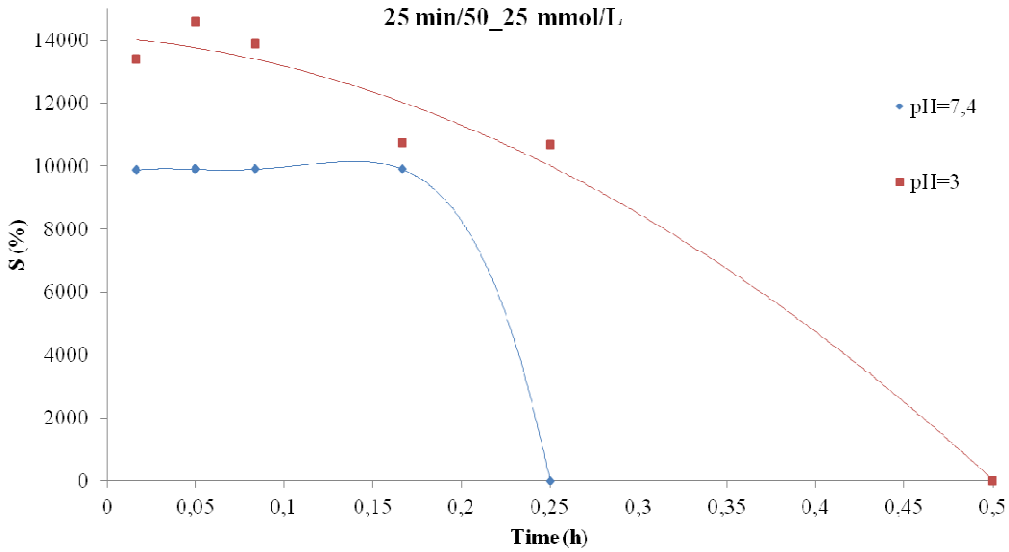
A 24 *Result of swelling test for sample crosslinked for 4 h in the solution of 50 mmol/L of EDC.*



A 25 *Result of swelling test for sample crosslinked for 8 h in the solution of 50 mmol/L of EDC.*

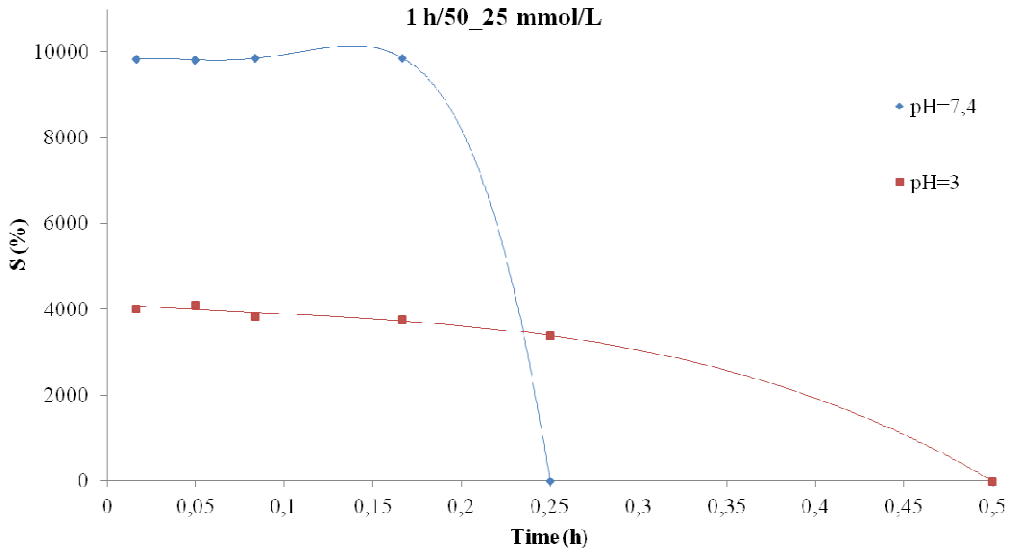


A 26 *Result of swelling test for sample crosslinked for 48 h in the solution of 50 mmol/L of EDC.*

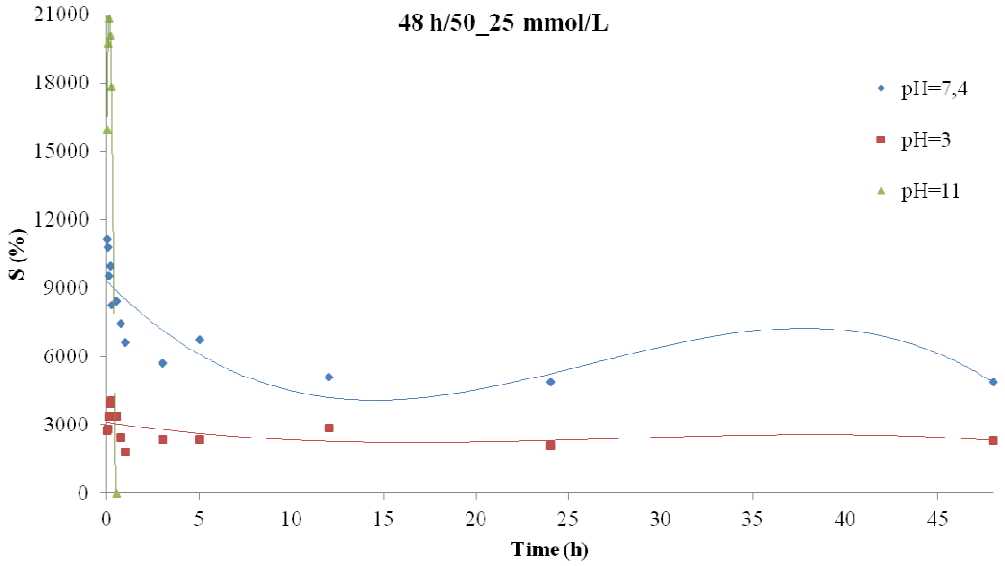


A 27 *Result of swelling test for sample crosslinked for 25 min in the solution of 50 mmol/L of EDC and 25 mmol/L of NHS.*

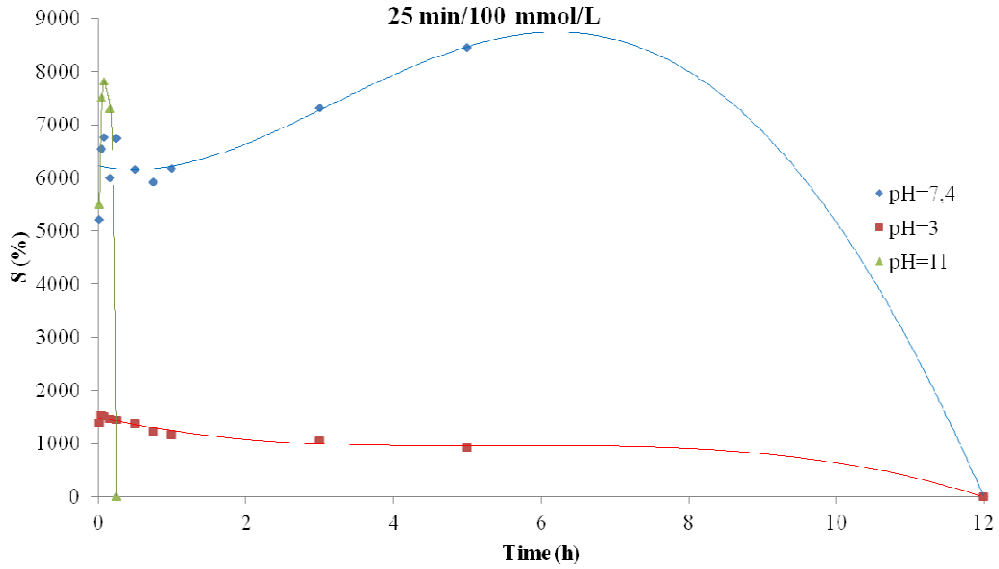
.



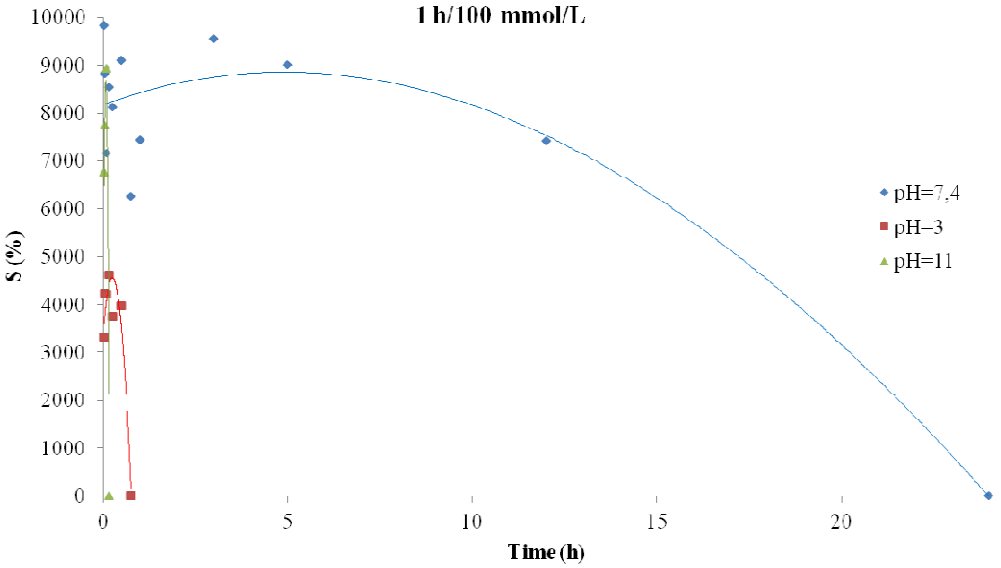
A 28 *Result of swelling test for sample crosslinked for 1 h in the solution of 50 mmol/L of EDC and 25 mmol/L of NHS.*



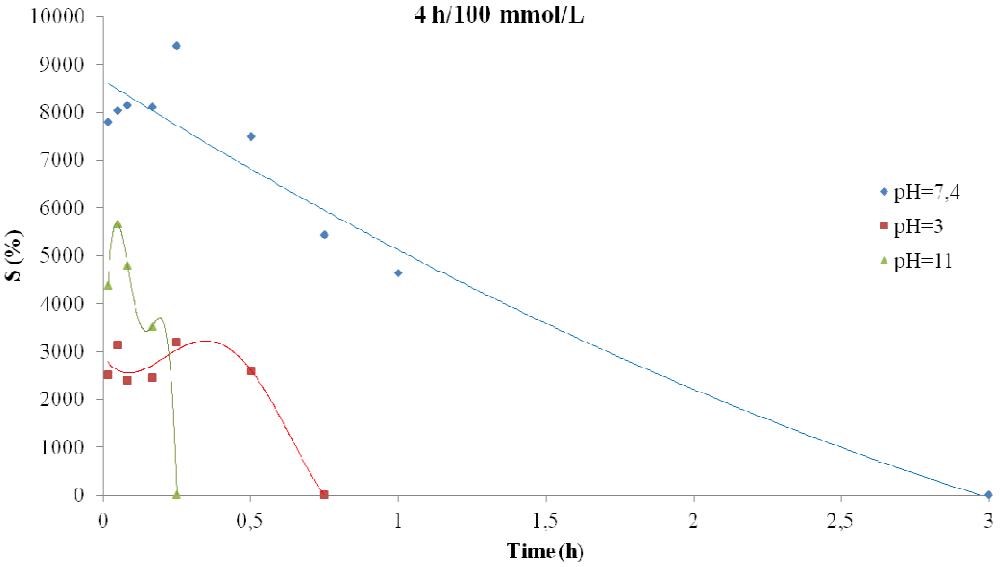
A 29 *Result of swelling test for sample crosslinked for 48 h in the solution of 50 mmol/L of EDC and 25 mmol/L of NHS.*



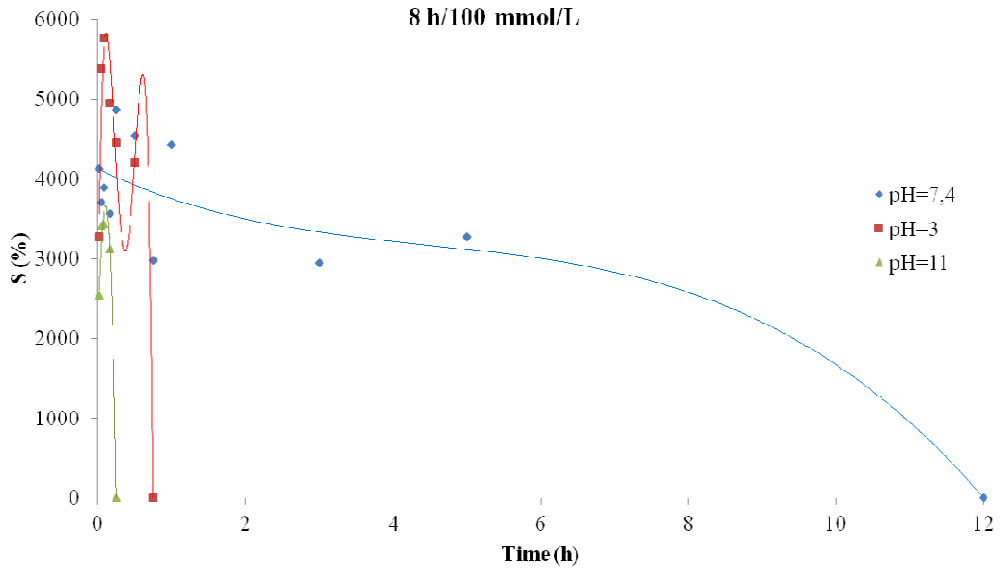
A 30 *Result of swelling test for sample crosslinked for 25 min in the solution of 100 mmol/L of EDC.*



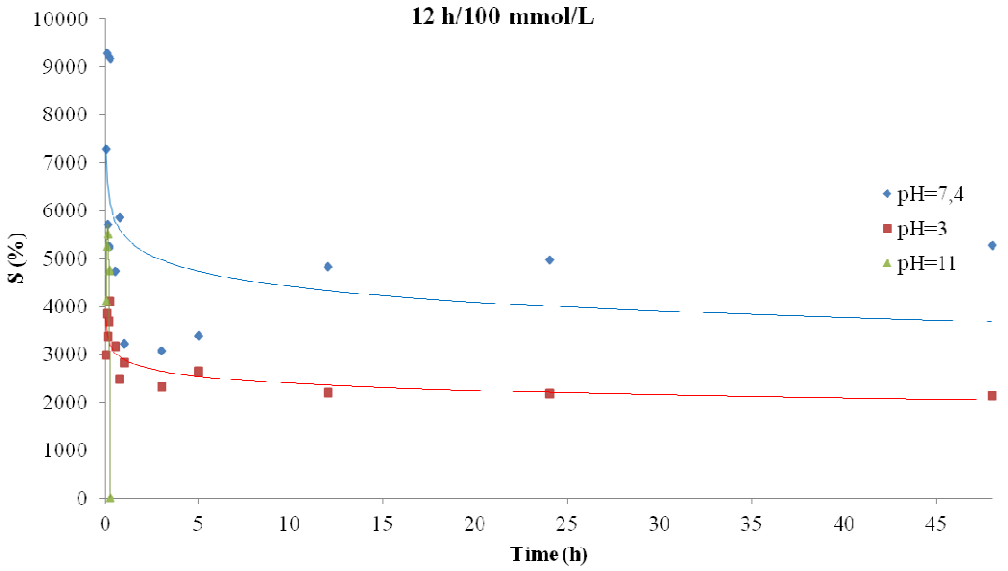
A 31 *Result of swelling test for sample crosslinked for 1 h in the solution of 100 mmol/L of EDC.*



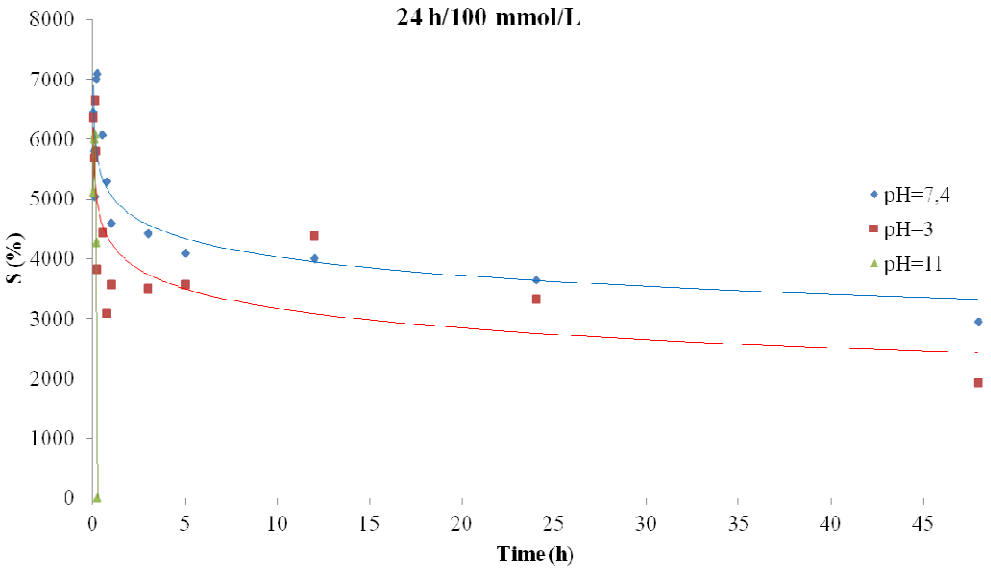
A 32 *Result of swelling test for sample crosslinked for 4 h in the solution of 100 mmol/L of EDC.*



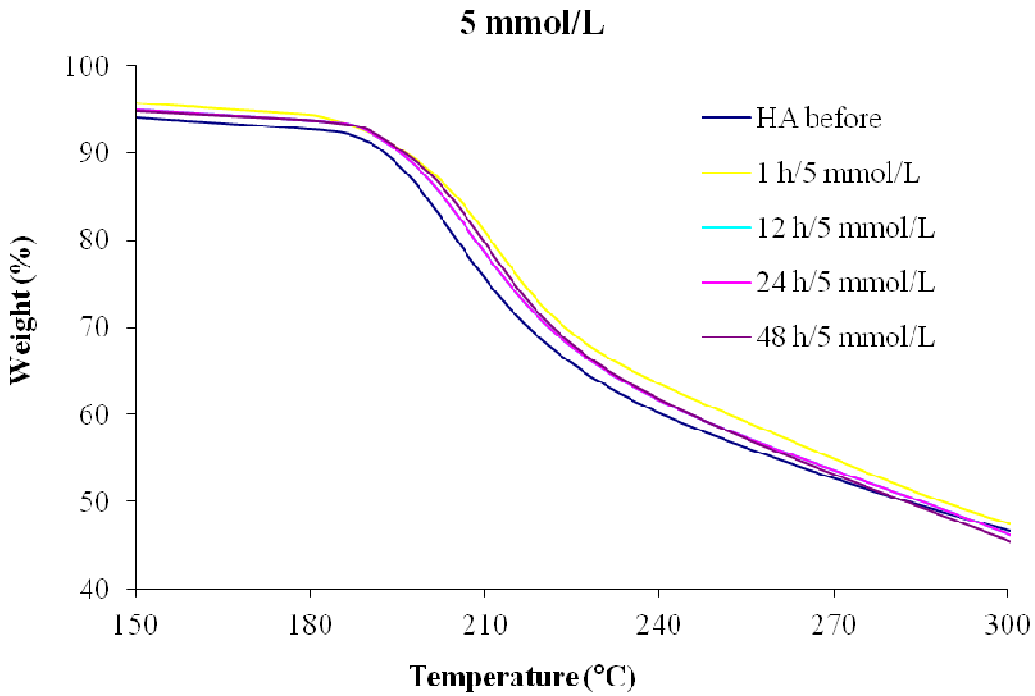
A 33 *Result of swelling test for sample crosslinked for 8 h in the solution of 100 mmol/L of EDC.*



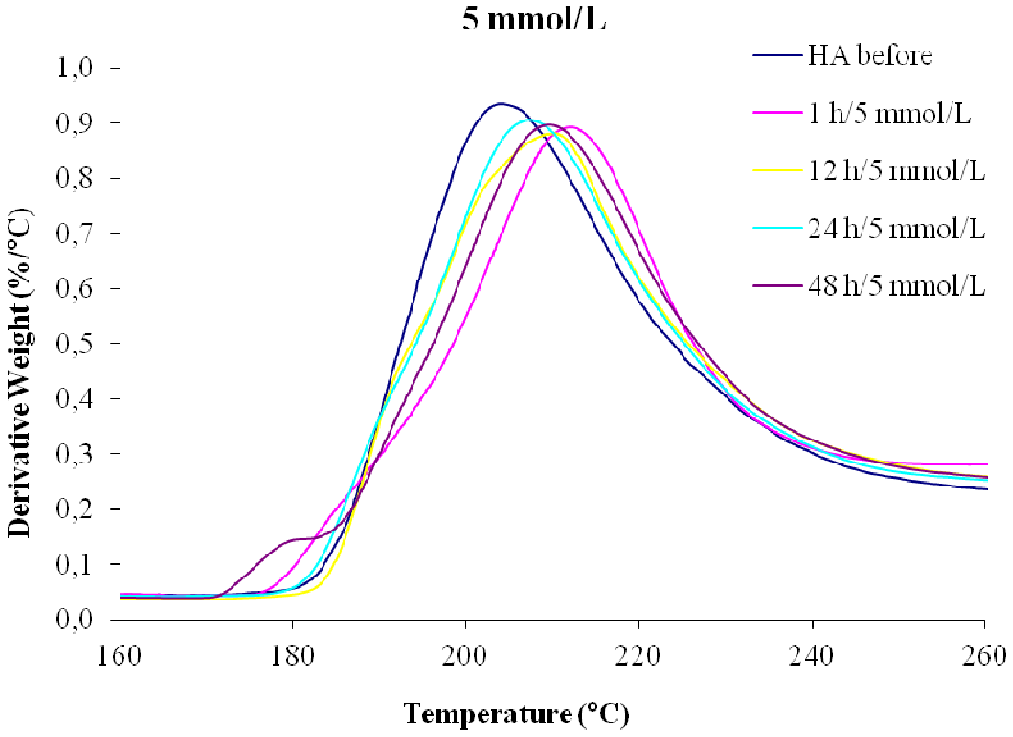
A 34 *Result of swelling test for sample crosslinked for 12 h in the solution of 100 mmol/L of EDC.*



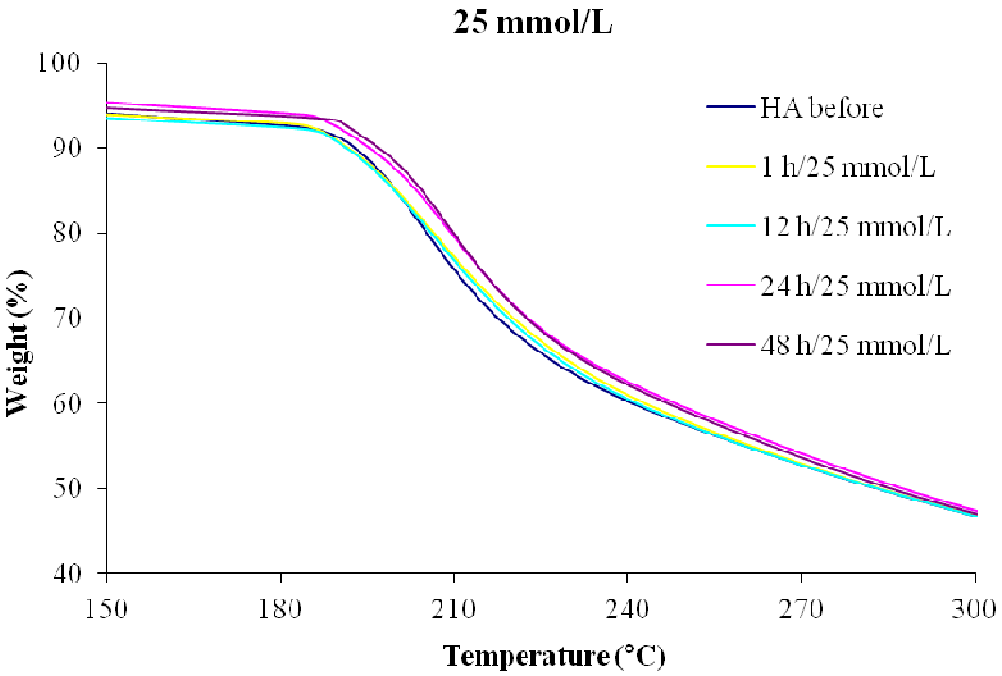
A 35 *Result of swelling test for sample crosslinked for 24 h in the solution of 100 mmol/L of EDC.*



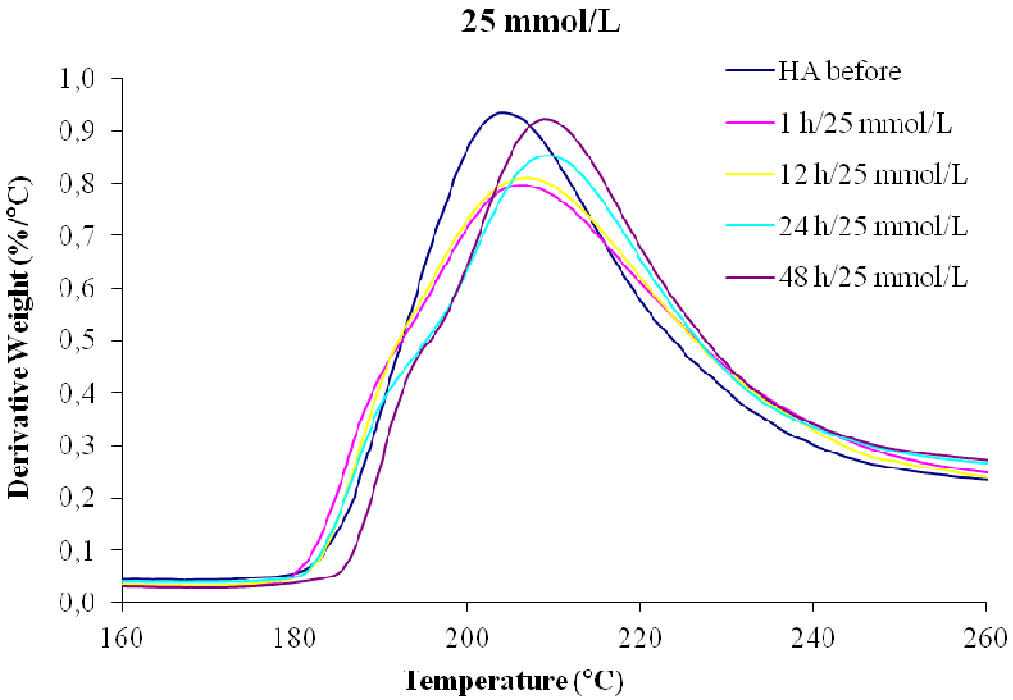
A 36 *Section of TGA curves for fibres crosslinked in solution with concentration 5 mmol/L of EDC.*



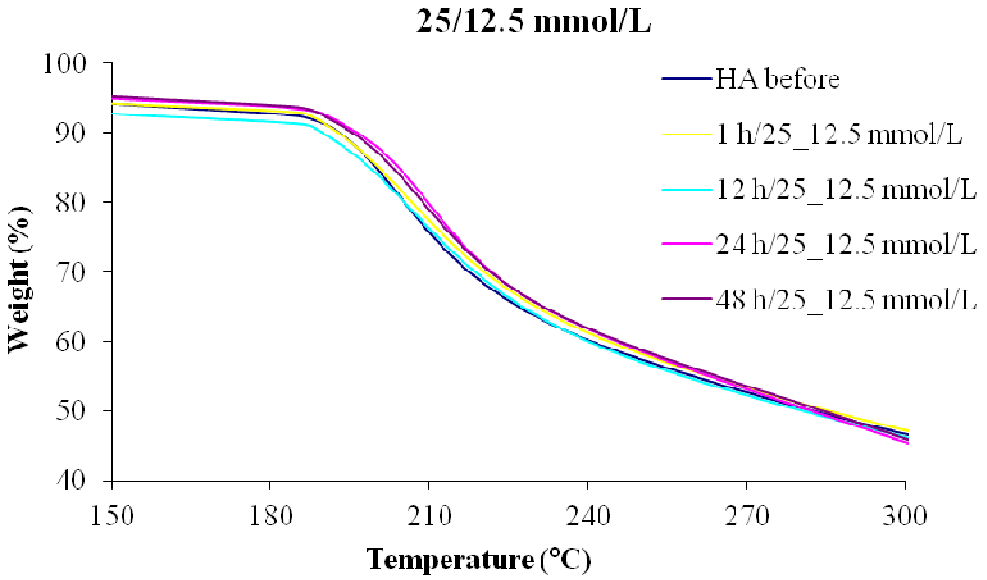
A 37 Section of the first derivation of TGA curves for fibres crosslinked in solution with concentration 5 mmol/L of EDC.



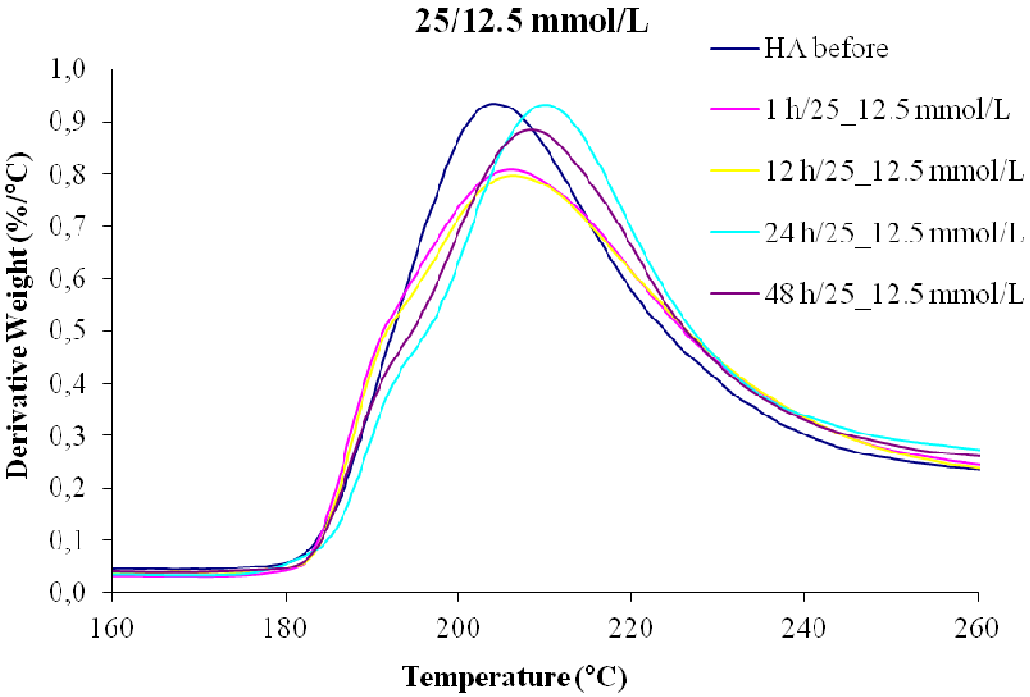
A 38 *Section of TGA curves for fibres crosslinked in solution with concentration 25 mmol/L of EDC.*



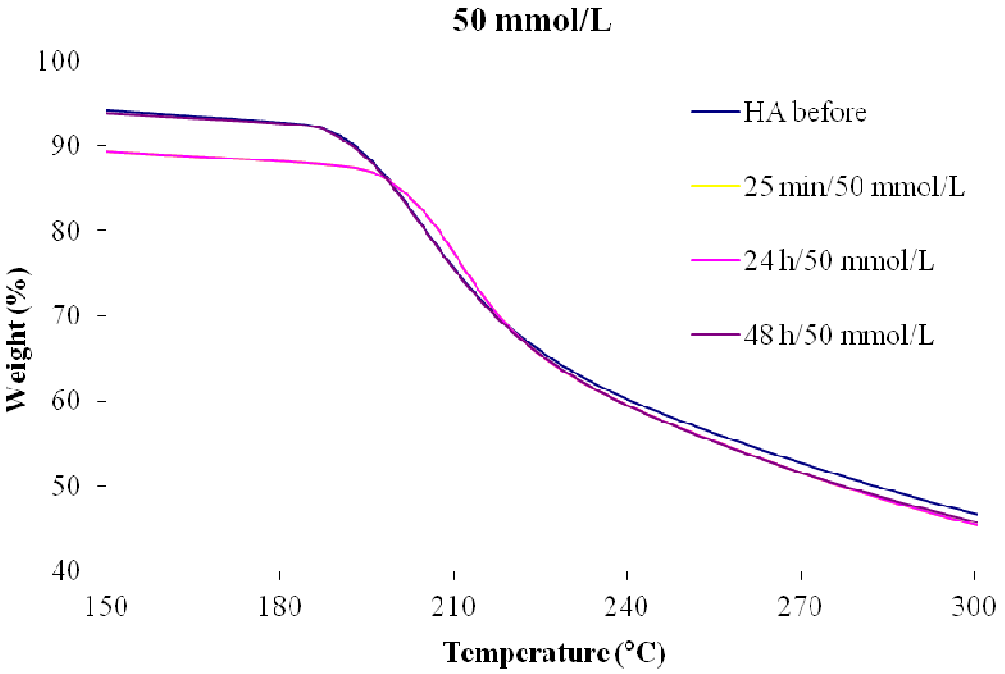
A 39 Section of the first derivation of TGA curves for fibres crosslinked in solution with concentration 25 mmol/L of EDC.



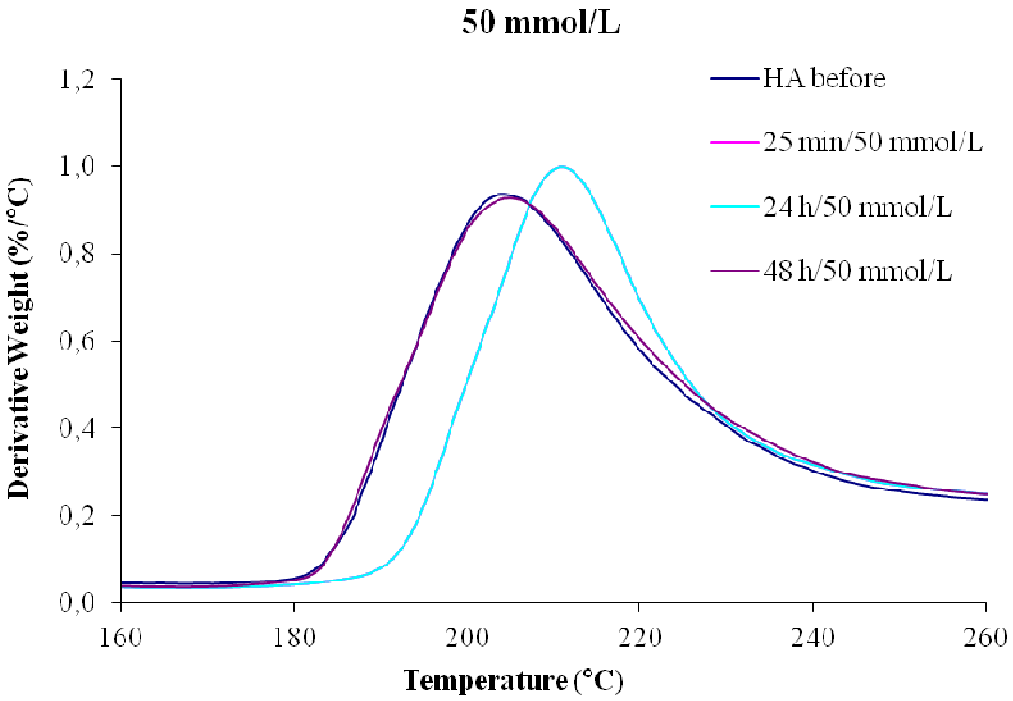
A 40 *Section of TGA curves for fibres crosslinked in solution with concentration 25 mmol/L of EDC and 12.5 mmol/L of NHS.*



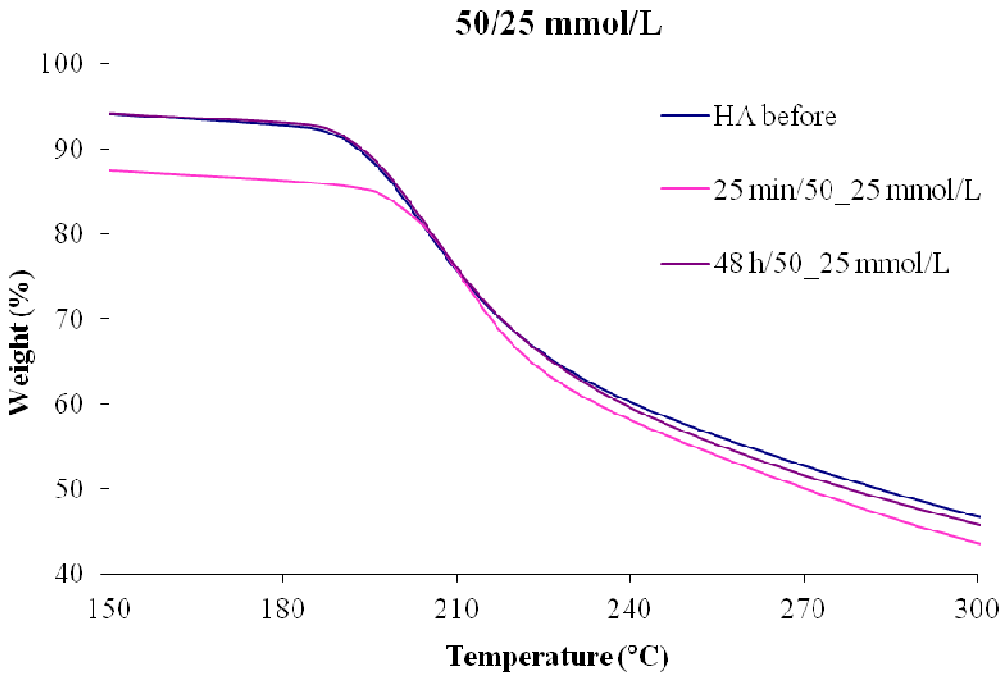
A 41 *Section of the first derivation of TGA curves for fibres crosslinked in solution with concentration 25 mmol/L of EDC and 12.5 mmol/L of NHS.*



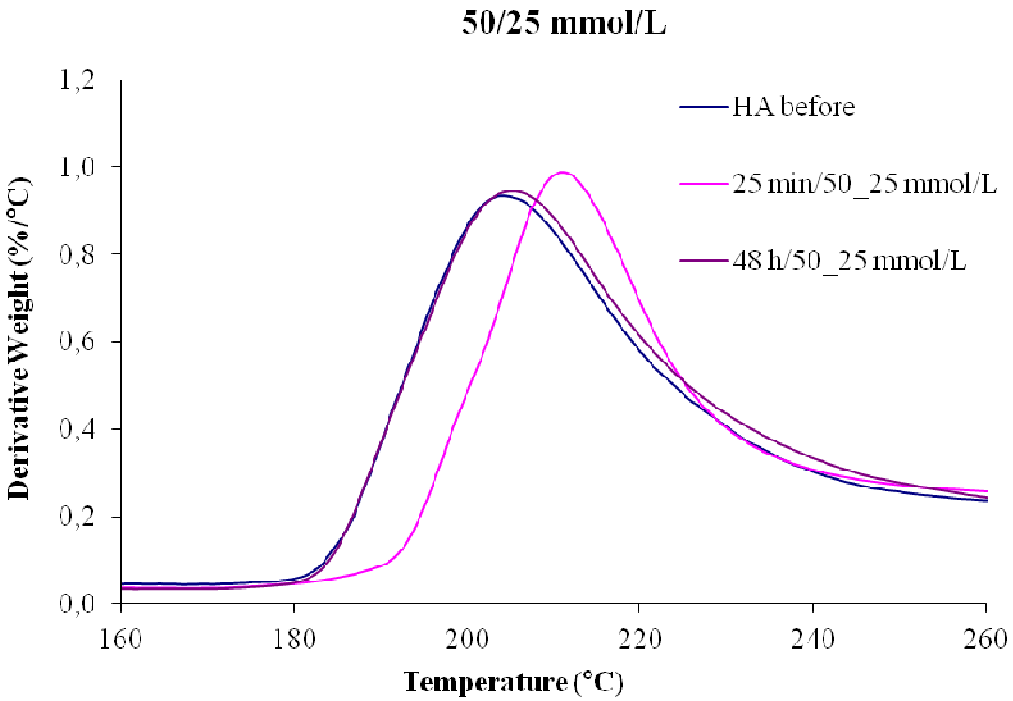
A 42 *Section of TGA curves for fibres crosslinked in solution with concentration 50 mmol/L of EDC.*



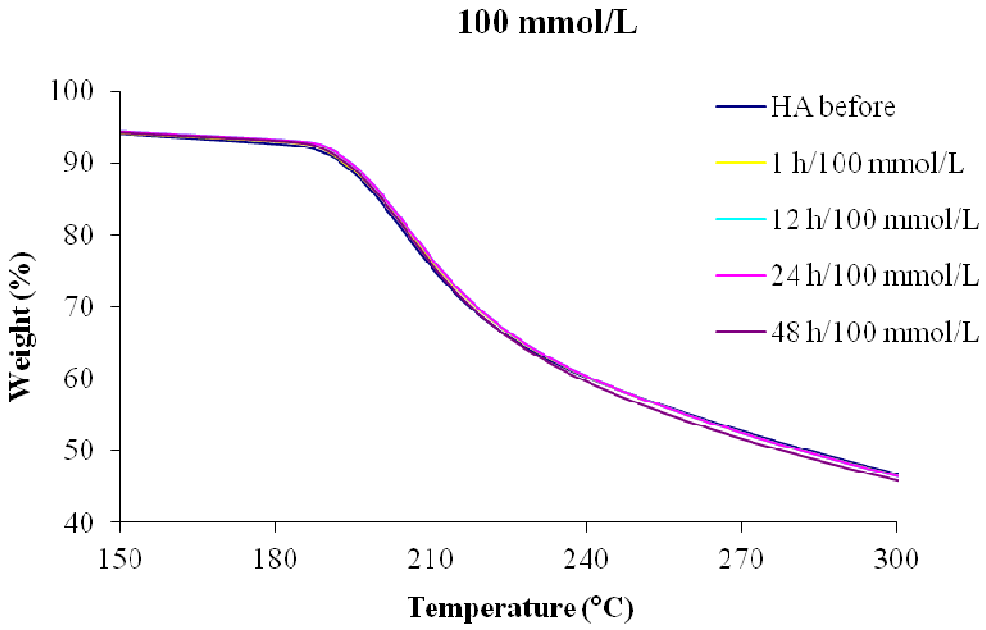
A 43 *Section of the first derivation of TGA curves for fibres crosslinked in solution with concentration 50 mmol/L of EDC.*



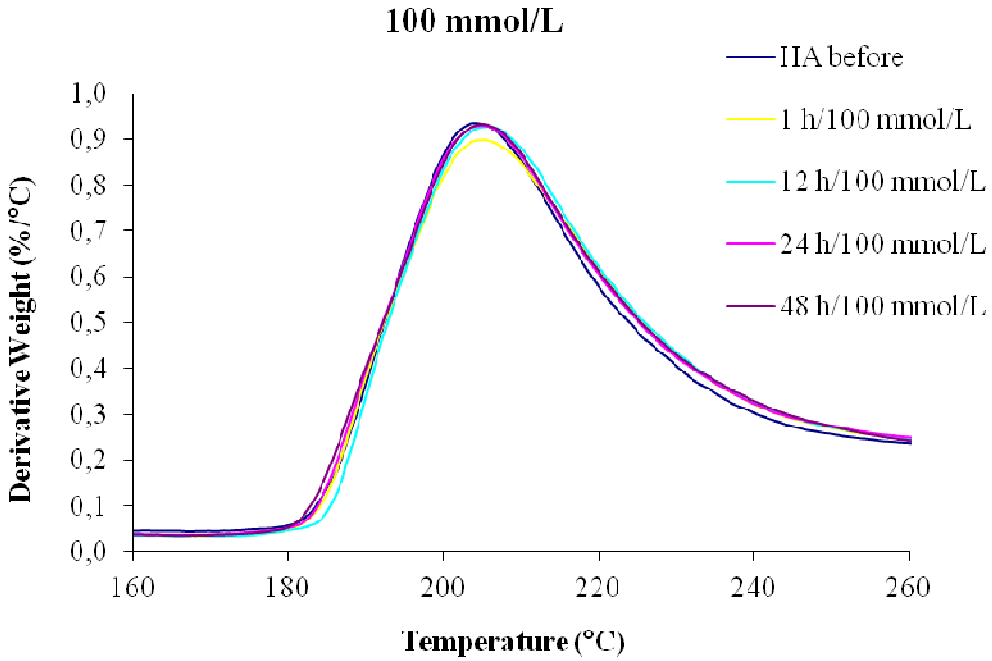
A 44 *Section of TGA curves for fibres crosslinked in solution with concentration 50 mmol/L of EDC and 25 mmol/L of NHS.*



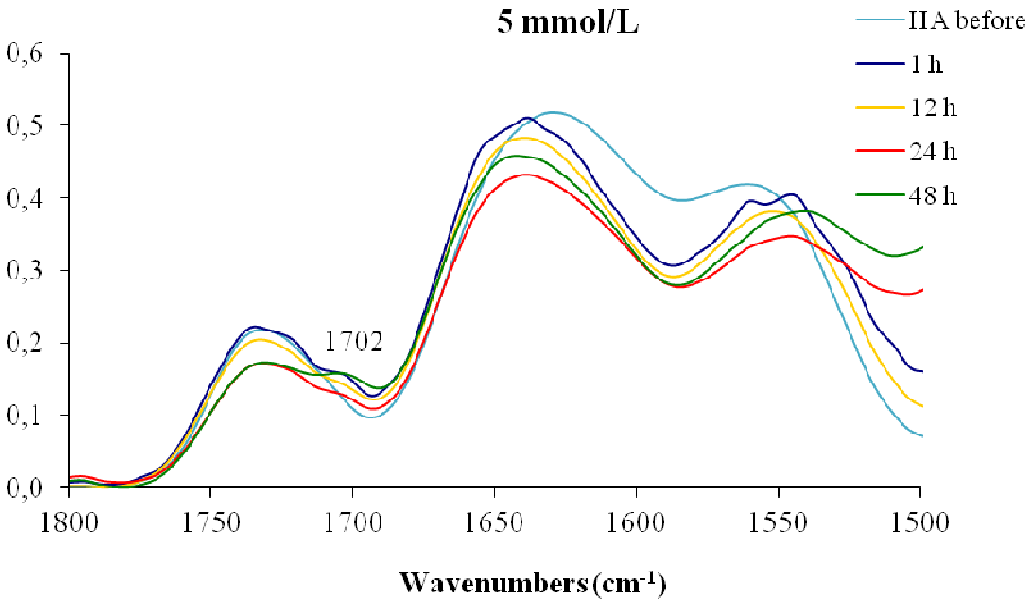
A 45 *Section of the first derivation of TGA curves for fibres crosslinked in solution with concentration 50 mmol/L of EDC and 25 mmol/L of NHS.*



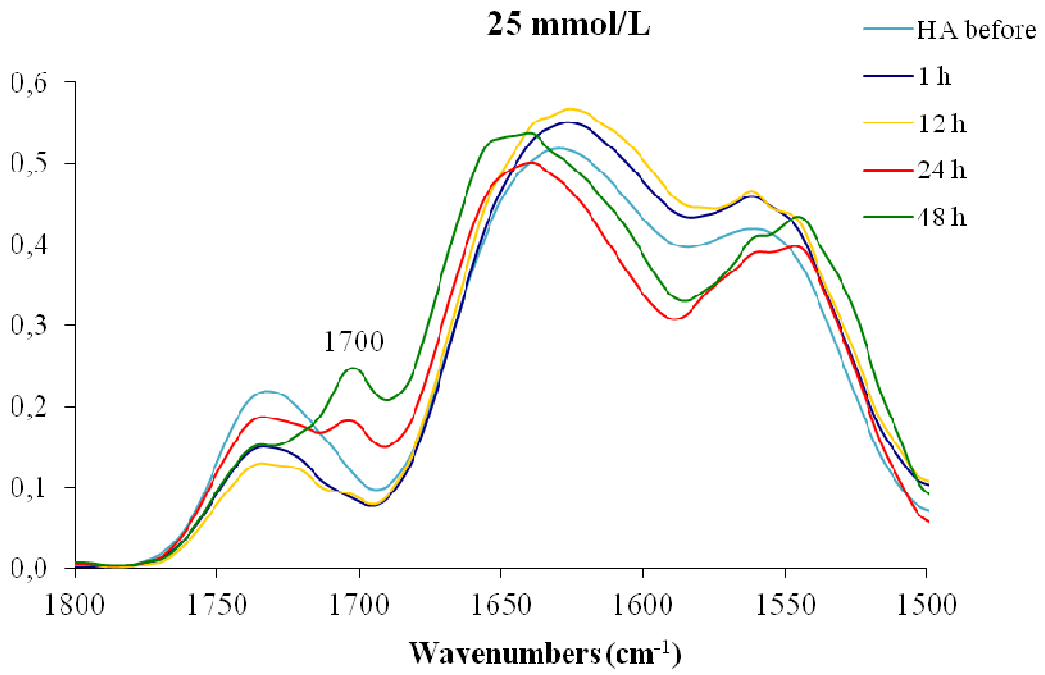
A 46 *Section of TGA curves for fibres crosslinked in solution with concentration 100 mmol/L of EDC.*



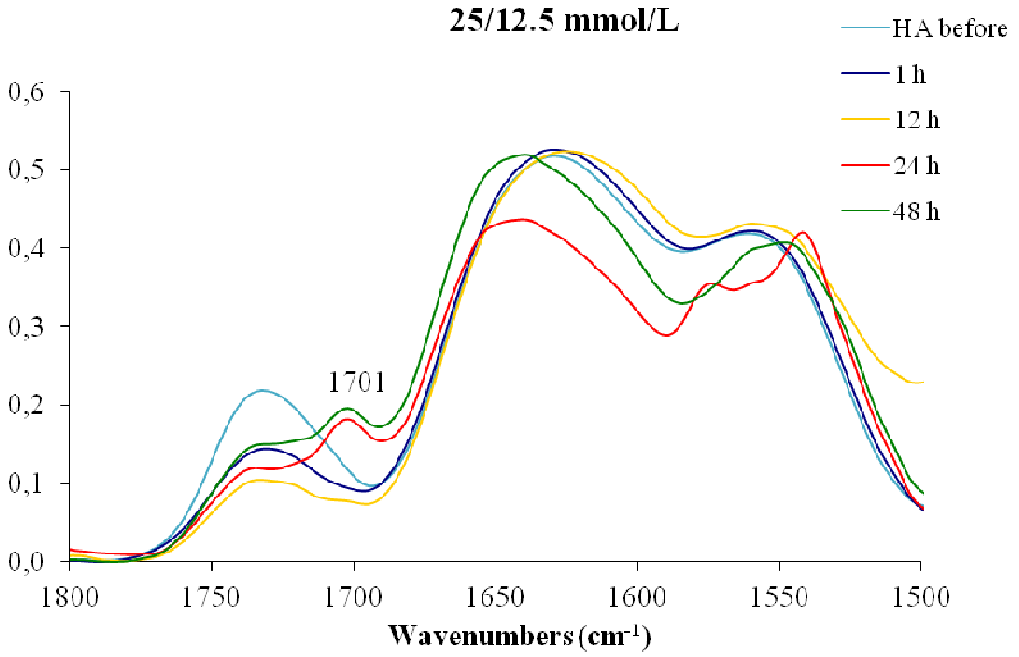
A 47 *Section of the first derivation of TGA curves for fibres crosslinked in solution with concentration 100 mmol/L of EDC.*



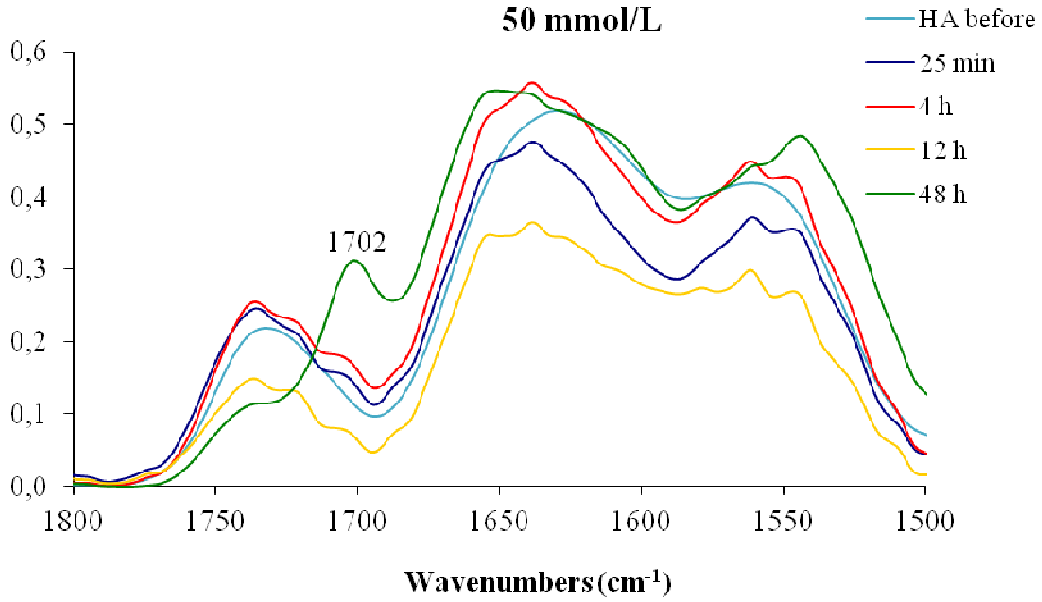
A 48 The section of infrared spectra comparing fibres crosslinked in the solution with concentration of 5 mmol/L EDC and hyaluronan fibre before crosslinking.



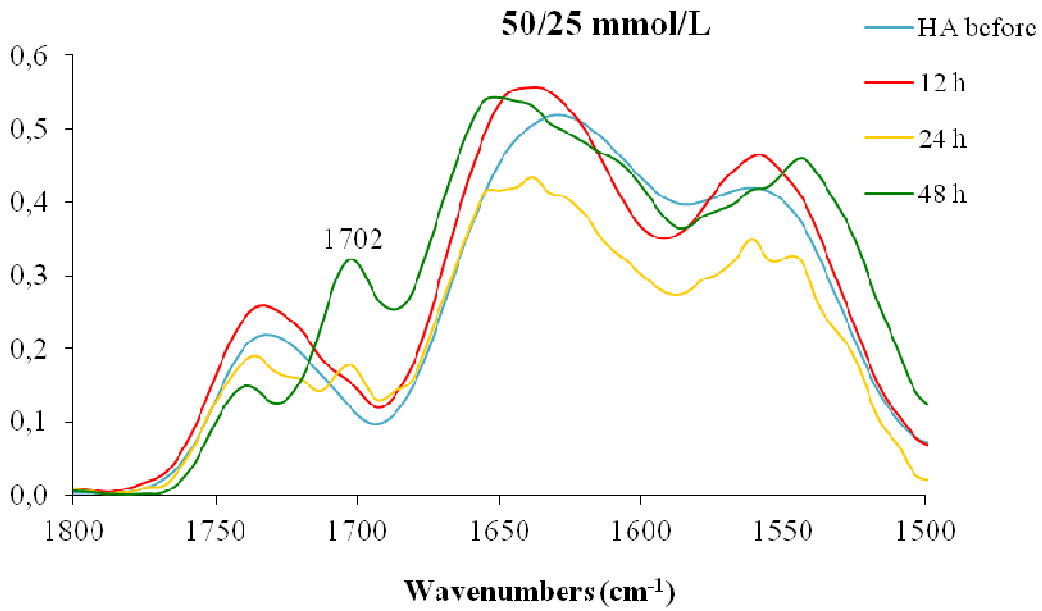
A 49 *The section of infrared spectra comparing fibres crosslinked in the solution with concentration of 25 mmol/L EDC and hyaluronan fibre before crosslinking.*



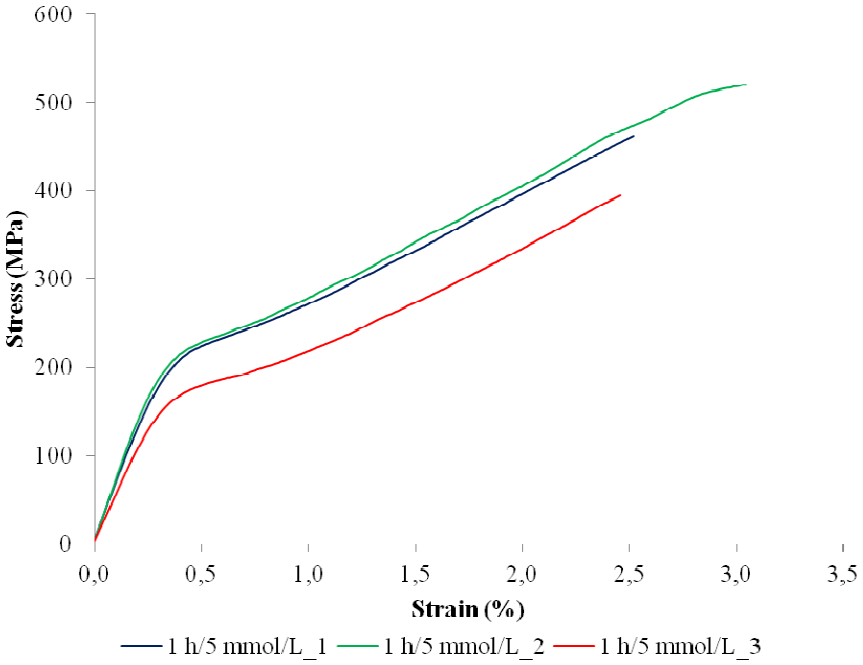
A 50 *The section of infrared spectra comparing fibres crosslinked in the solution with concentration of 25 mmol/L EDC and 12.5 mmol/L NHS and hyaluronan fibre before crosslinking.*



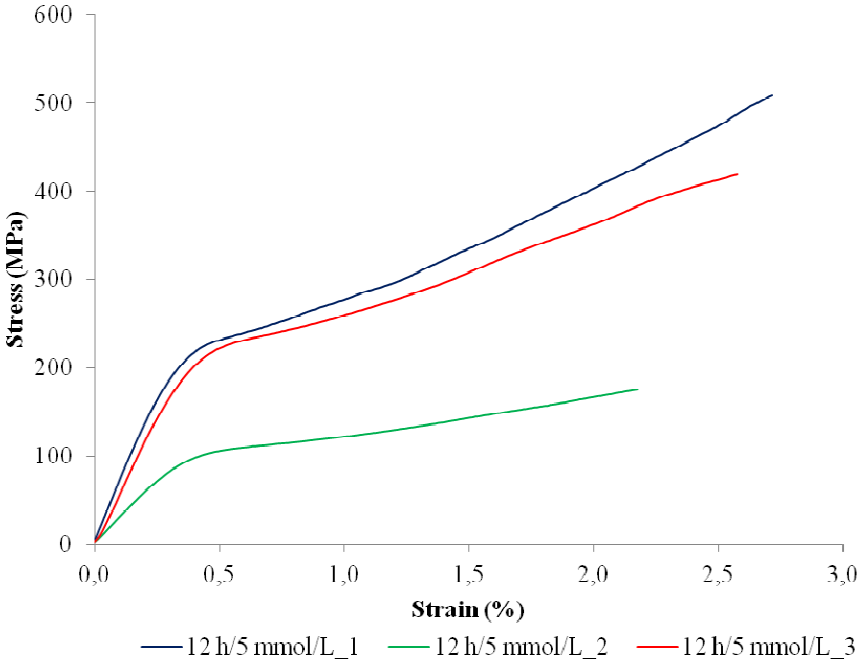
A 51 *section of infrared spectra comparing fibres crosslinked in the solution with concentration of 50 mmol/L EDC and hyaluronan fibre before crosslinking.*



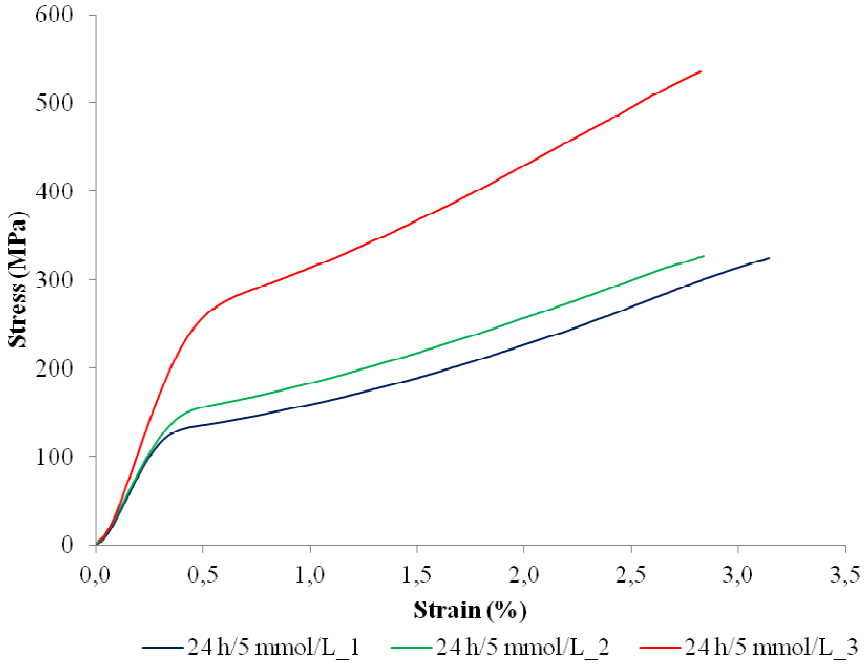
A 52 *The section of infrared spectra comparing fibres crosslinked in the solution with concentration of 50 mmol/L EDC and 25 mmol/L NHS and hyaluronan fibre before crosslinking.*



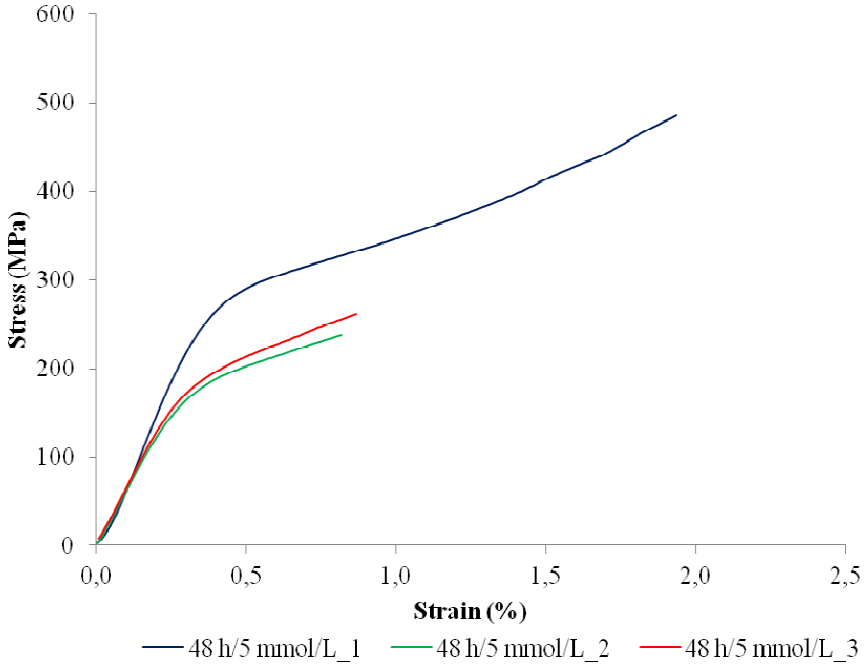
A 53 *The stress-strain curves of fibres crosslinked for 1 h in solution with concentration of 5 mmol/L EDC.*



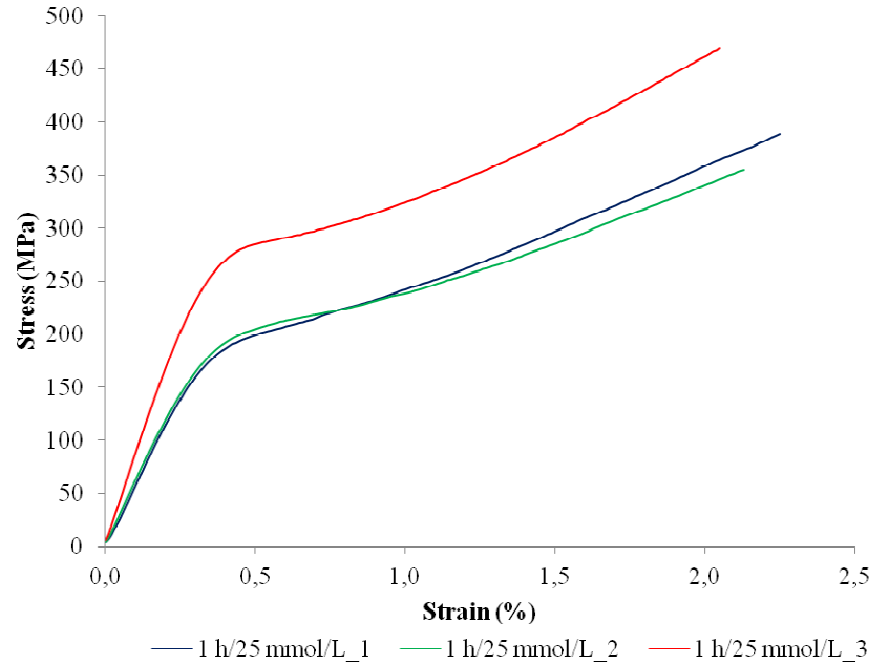
A 54 *The stress-strain curves of fibres crosslinked for 12 h in solution with concentration of 5 mmol/L EDC.*



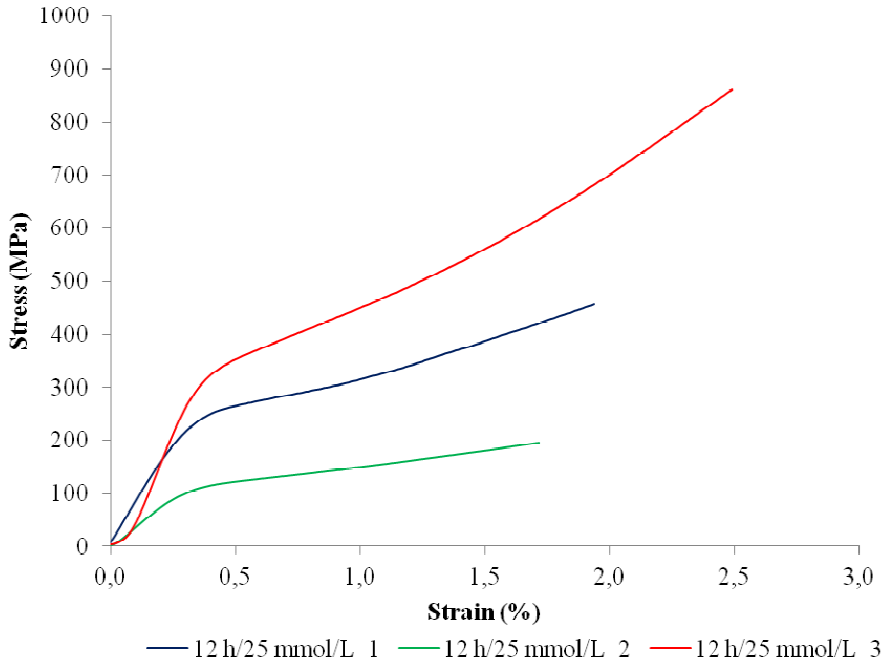
A 55 *The stress-strain curves of fibres crosslinked for 24 h in solution with concentration of 5 mmol/L EDC.*



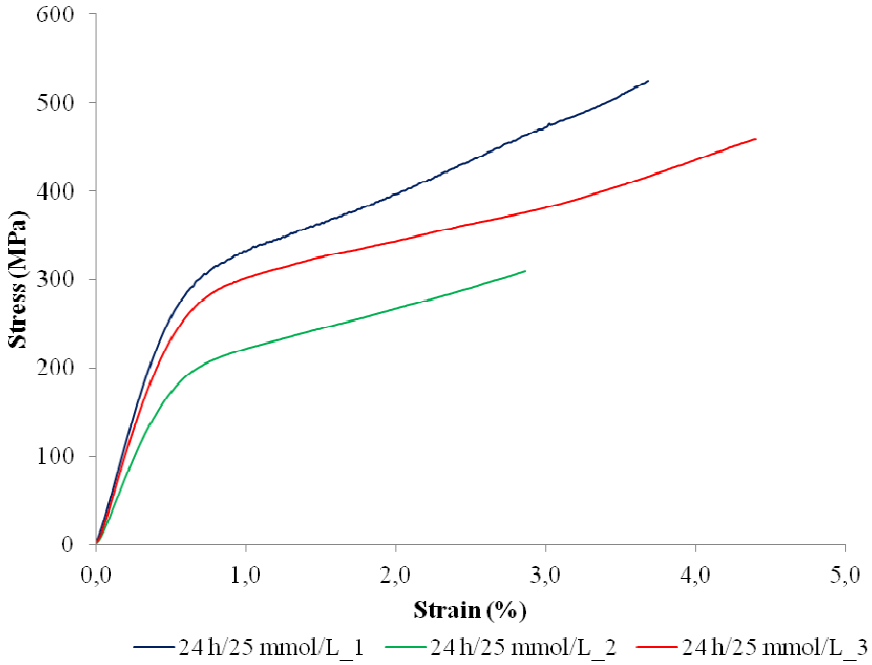
A 56 *The stress-strain curves of fibres crosslinked for 48 h in solution with concentration of 5 mmol/L EDC.*



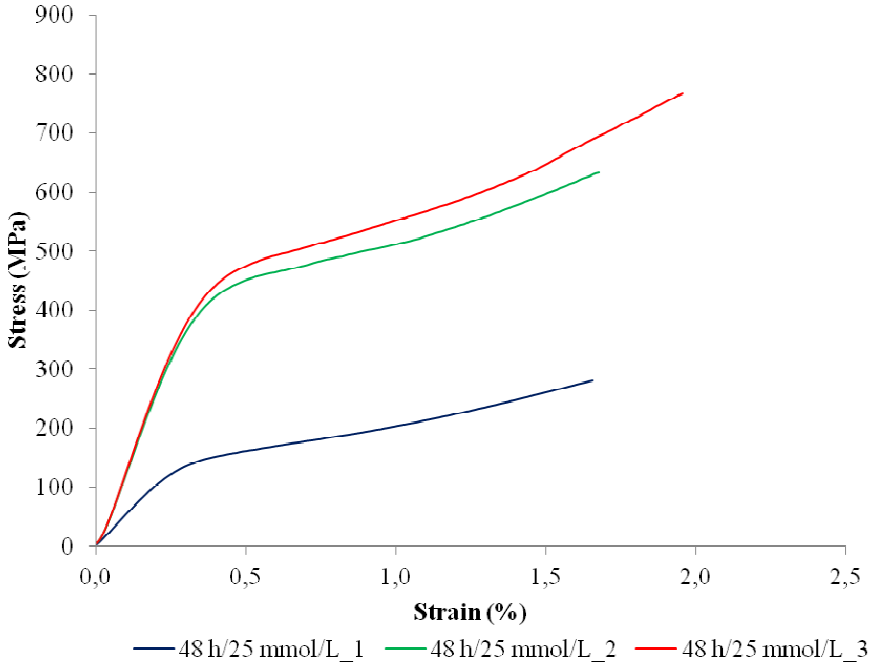
A 57 *The stress-strain curves of fibres crosslinked for 1 h in solution with concentration of 25 mmol/L EDC.*



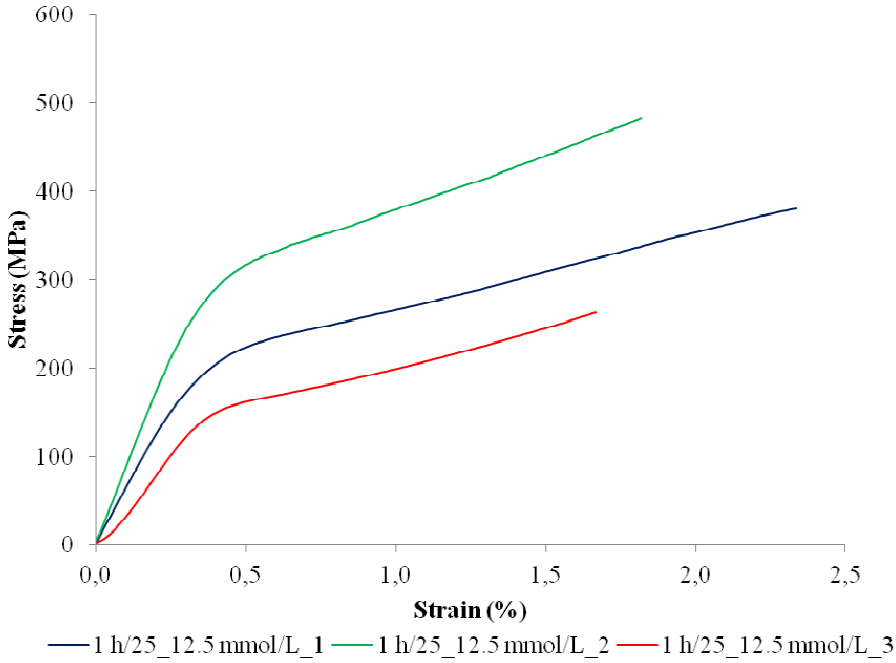
A 58 *The stress-strain curves of fibres crosslinked for 12 h in solution with concentration of 25 mmol/L EDC.*



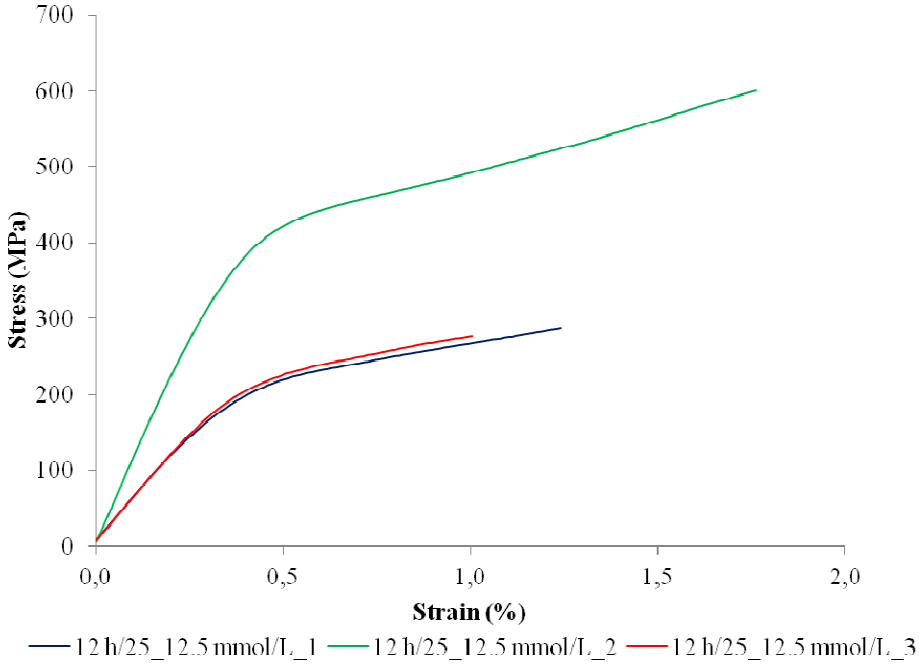
A 59 *The stress-strain curves of fibres crosslinked for 24 h in solution with concentration of 25 mmol/L EDC.*



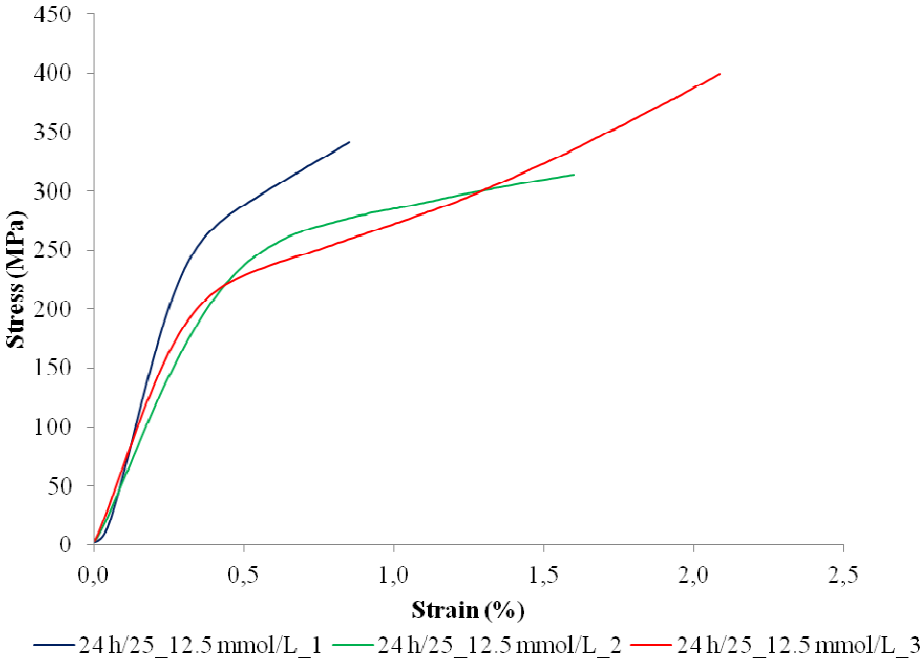
A 60 *The stress-strain curves of fibres crosslinked for 48 h in solution with concentration of 25 mmol/L EDC.*



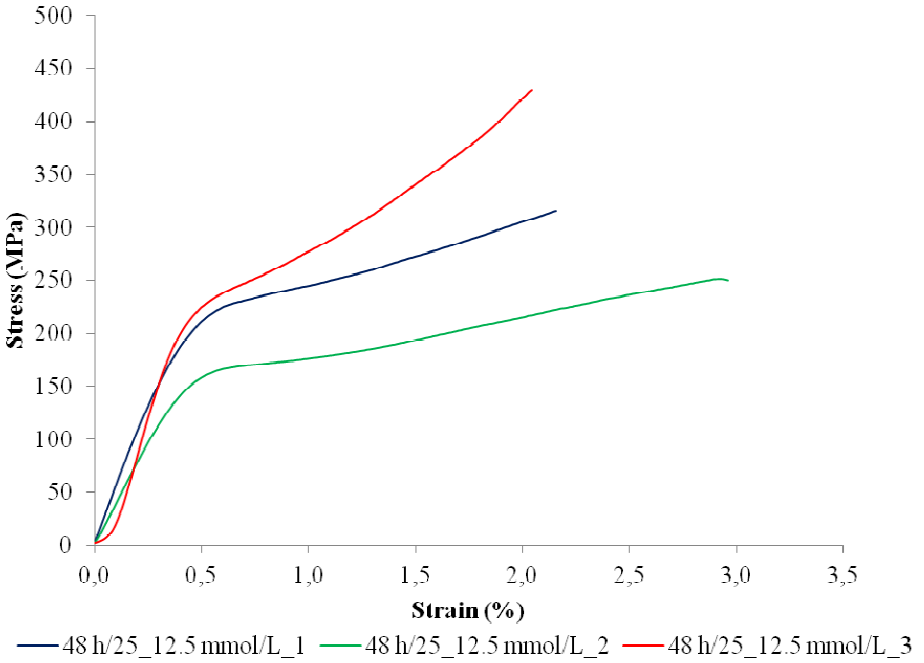
A 61 *The stress-strain curves of fibres crosslinked for 1 h in solution with concentration of 25 mmol/L EDC and 12.5 mmol/L NHS.*



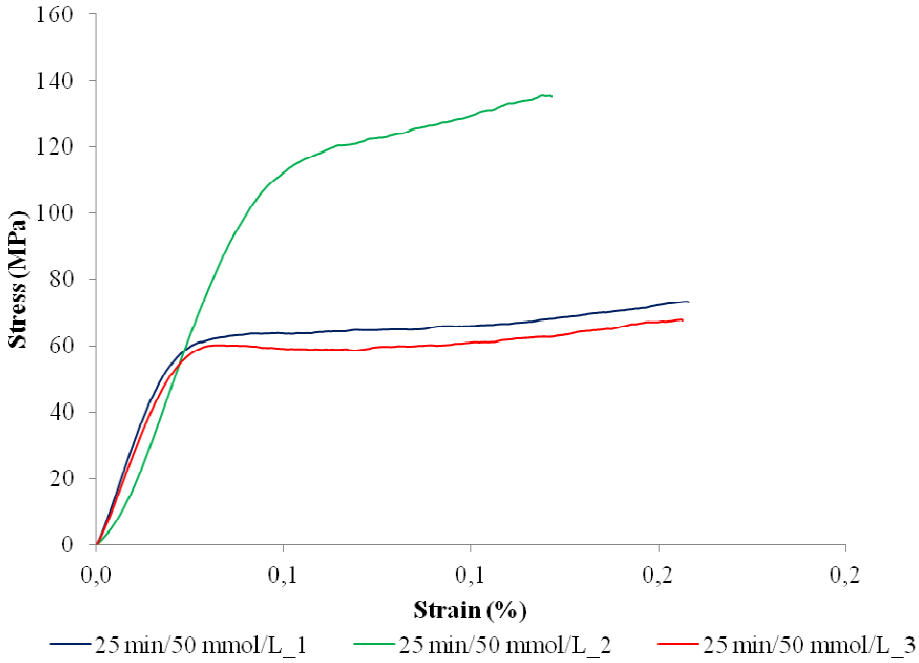
A 62 *The stress-strain curves of fibres crosslinked for 12 h in solution with concentration of 25 mmol/L EDC and 12.5 mmol/L NHS.*



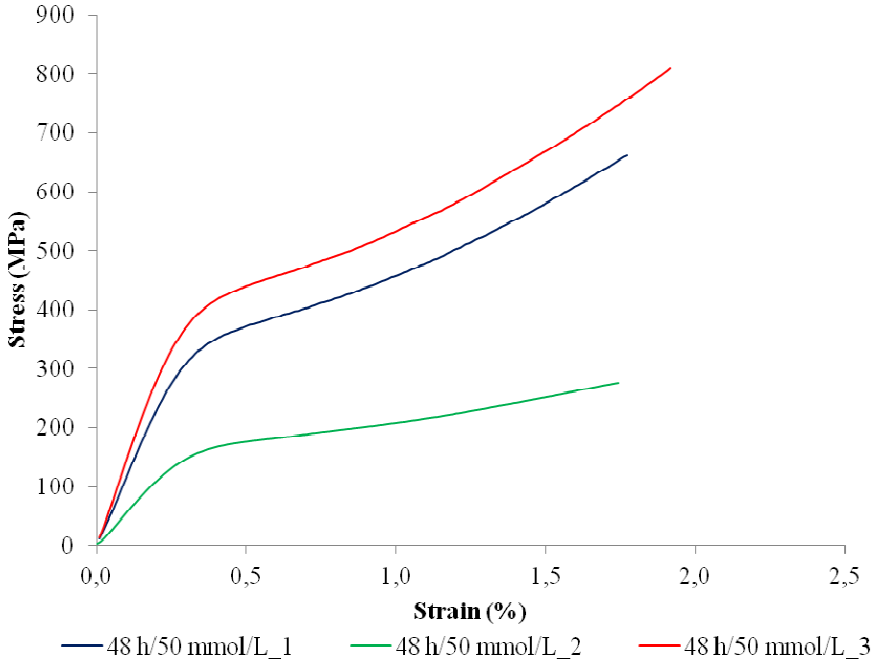
A 63 *The stress-strain curves of fibres crosslinked for 24 h in solution with concentration of 25 mmol/L EDC and 12.5 mmol/L NHS.*



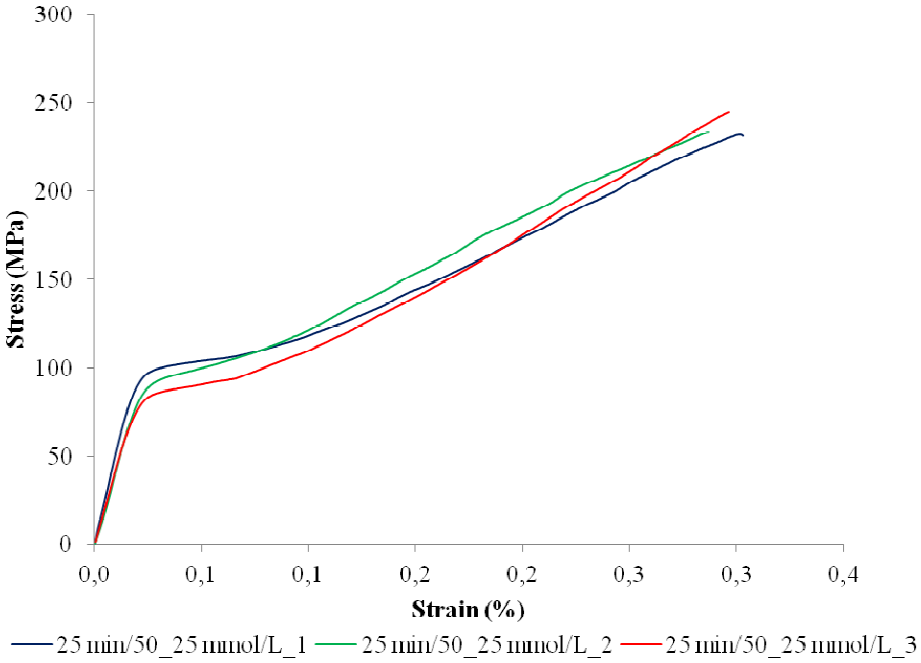
A 64 *The stress-strain curves of fibres crosslinked for 48 h in solution with concentration of 25 mmol/L EDC and 12.5 mmol/L NHS.*



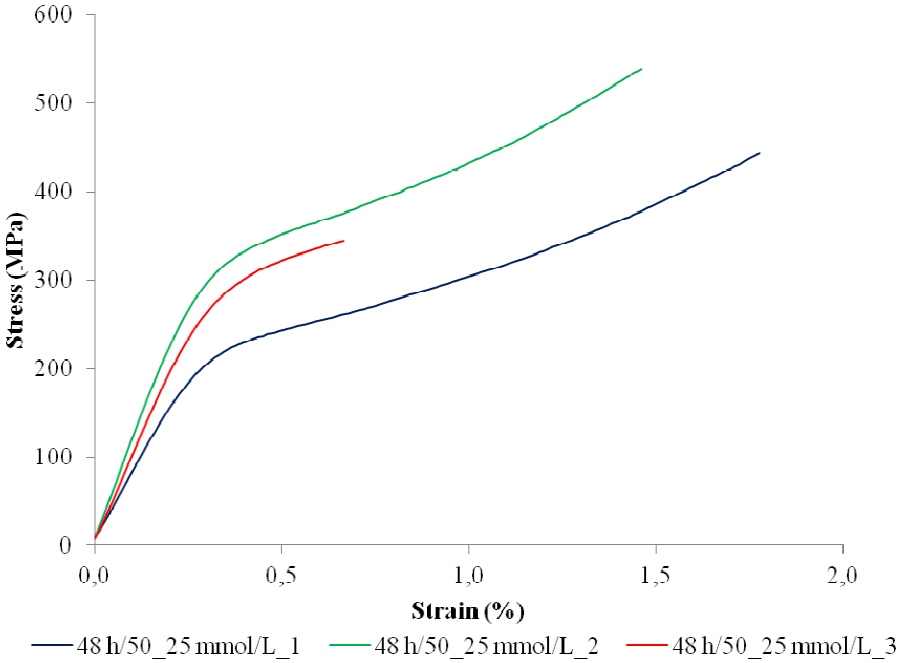
A 65 *The stress-strain curves of fibres crosslinked for 25 min in solution with concentration of 50 mmol/L EDC.*



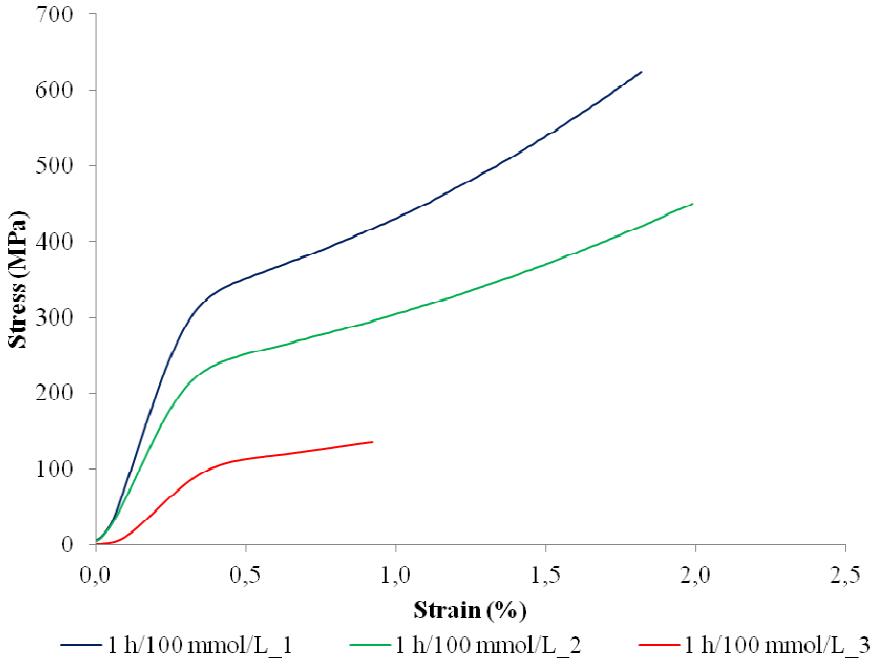
A 66 *The stress-strain curves of fibres crosslinked for 48 h in solution with concentration of 50 mmol/L EDC.*



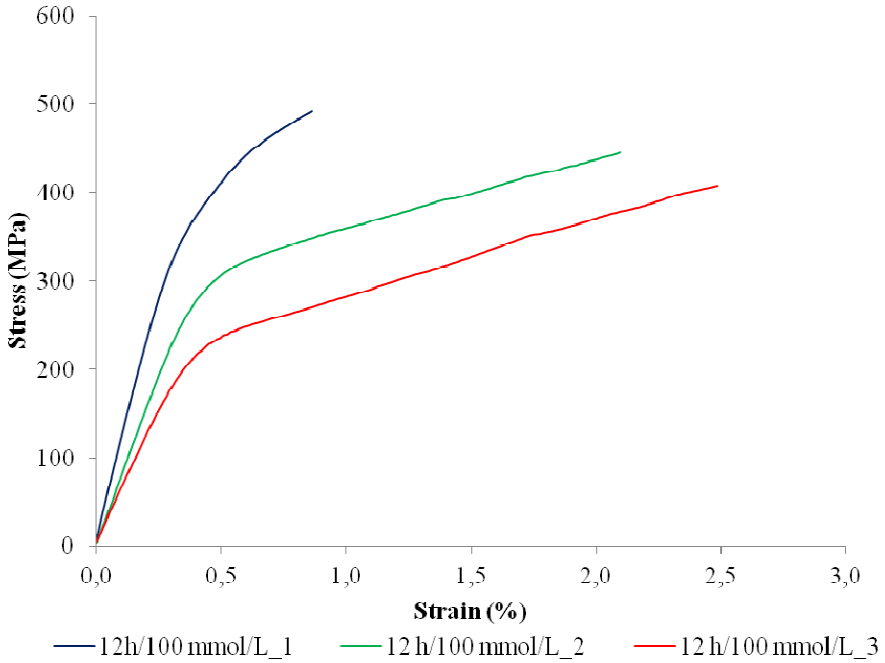
A 67 *The stress-strain curves of fibres crosslinked for 25 min in solution with concentration of 50 mmol/L EDC and 25 mmol/L NHS.*



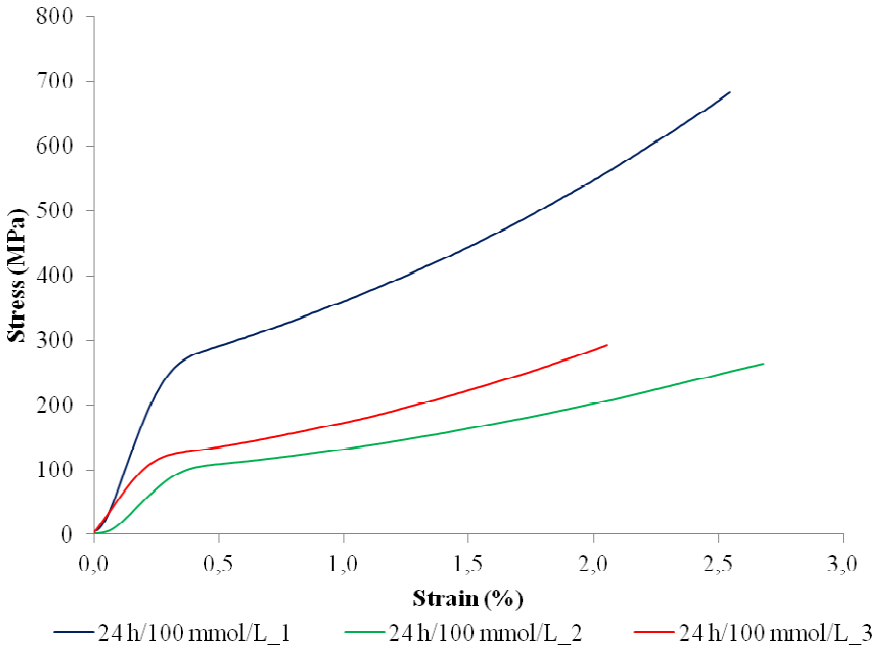
A 68 *The stress-strain curves of fibres crosslinked for 48 h in solution with concentration of 50 mmol/L EDC and 25 mmol/L NHS.*



A 69 *The stress-strain curves of fibres crosslinked for 1 h in solution with concentration of 100 mmol/L EDC.*



A 70 *The stress-strain curves of fibres crosslinked for 12 h in solution with concentration of 100 mmol/L EDC.*



A 71 *The stress-strain curves of fibres crosslinked for 24 h in solution with concentration of 100 mmol/L EDC.*